



Structure-function studies of β -lactamases

Agustin Zavala

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Structure-function studies of β -lactamases

Thèse de doctorat de l'Université Paris-Saclay

Préparée à la Faculté de Pharmacie, Université Paris-Sud.

École doctorale n°569

Innovation thérapeutique : du fondamental à l'appliqué.

Spécialité de doctorat: Microbiologie et Thérapeutiques anti-infectieuses

Thèse présentée et soutenue à Le Kremlin-Bicêtre, le 19 décembre 2018

Agustin Zavala

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Acronyms

6-APA	6-aminopenicillanic acid
7-ACA	7-aminocephalosporanic acid
AA	amino acid
ABC	ATP-Binding Cassette transporters
ACE	angiotensin converting enzyme
AMA	aspergillomarasmine A
AMR	antimicrobial resistance
ATB	antibiogram
BL	β -lactamase
BLDB	β -lactamase data base
BTZ	bisthiazolidines
CHDL	carbapenem-hydrolyzing class D β -lactamase
DABCO	diazabicyclooctanone
DBL	class D β -lactamase
DBO	diazabicyclo-octanones
DHP	dihidropetidase
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ESAC	extended-spectrum AmpC
ESBL	extended-spectrum β -lactamase
FDA	Food and Drug Administration
HMM	high molecular mass
HTS	high throughput screening

IC50	half maximal inhibitory concentration
IM	inner membrane
IS	insertion sequence
LMM	low molecular mass
LPS	lipopolysaccharide
MATE	Multidrug and Toxic Compound Extrusion
MBL	metallo- β -lactamase
MCSS	Multiple Copy Simultaneous Search
MD	Molecular Dynamics Simulation
MFS	Major Facilitator Superfamily
MIC	minimum inhibitory concentration
MLST	multi locus sequence typing
MRSA	methicillin resistant <i>S. aureus</i>
NAG	N-acetylglucosamine
NAM	N-actyl muramic acid
NCBI	National Center for Biotechnology Information
NCR	national reference center
OM	outer membrane
OMP	outer membrane protein
PDB	Protein Data Bank
PBP	penicillin-binding proteins
QM/MM	quantum mechanics/molecular mechanics
RMSD	root-mean-square deviation
RNA	ribonucleic acid

RND	Resistance Nodulation cell Division
SBL	serine- β -lactamase
SCOP	Structural Classifications of Proteins database
SMR	Small Multidrug Resistance
TDR	totally-drug resistance
TLS	Translation/Libration/Screw
XDR	extensively-drug resistance

Introduction

Background:

Antimicrobial resistance has become a major threat to public health challenges nowadays. The use and abuse of antibiotics is leading to the increasing selection of resistance mechanisms worldwide, to even the latest and most potent antimicrobial drugs available. The spread and acquisition of resistance mechanisms has led to multi drug resistant pathogens, greatly compromising our capacity to treat infectious diseases. Antibiotic resistance might ultimately result in a future without effective antimicrobial therapy.

According to O'Neill's report (<https://amr-review.org>), without policies to stop the spread of antimicrobial resistance (AMR), infectious diseases deaths may reach 10 million per year in 2050, surpassing cancer-related deaths. Apart from the human lives cost, AMR represents a real economic cost to society, which will continue to increase if AMR is not dealt with. Globally, economic losses between now and 2050 are estimated to be over 100 trillion USD.

Several measures must be taken to tackle this problem: i) massive global public awareness campaign; ii) hygiene improvement and prevention of the spread of infections; iii) reduction of unnecessary use of antimicrobials in agriculture and their dissemination into the environment; iv) improvement of global surveillance of drug resistance and antimicrobial consumption in humans and animals; v) development of new, rapid diagnostic methods to provide appropriate treatment and reduce the unnecessary use of antibiotics; vi) promotion of development and use of vaccines and alternative therapies.

Development of new antimicrobial drugs, for either already known or novel targets, is also part of the strategy. A related approach is the development of inhibitors that target the resistance mechanisms. The advantage behind this last possibility is that they may allow us to continue using many of the antimicrobial drugs that have already been developed and are known to be safe for humans. In order to do this, better understanding the different resistance mechanisms would probably be very useful. Thus, efforts to elucidate the behaviour of enzymes conferring resistance to MDR bacteria may ultimately prove extremely helpful in dealing with the AMR threat.

Enterobacteriaceae:

A) General characteristics:

The Enterobacteriaceae family comprises species that are human pathogens (e.g. *Salmonella enterica*, *Shigella sp.* *Yersinia sp.*) as well as species belonging to normal mammalian gut microbiota that are opportunistic pathogens (e.g. *Escherichia coli*, *Klebsiella spp.*, *Proteus spp.*). They are Gram-negative bacilli, facultative anaerobes, and sugar fermenting. Except for a few genera, most possess flagella and are motile. They do not form spores, and catalase reaction results vary among Enterobacteriaceae. Most members possess type I fimbriae to adhere to their hosts cells. Enterobacteriaceae genome GC% varies between 39 and 59%.

B) Cell Envelope:

Unlike Gram-positive bacteria, the cell envelope of Gram-negative bacteria is always composed of at least three layers: the cytoplasmic, or inner, membrane (IM); the peptidoglycan, or murein, cell wall; and the outer membrane (OM). The two lipidic membranes delimit an aqueous compartment named periplasm, where the thin layer of peptidoglycan is found [Mitchell, 1961] (Figure 1).

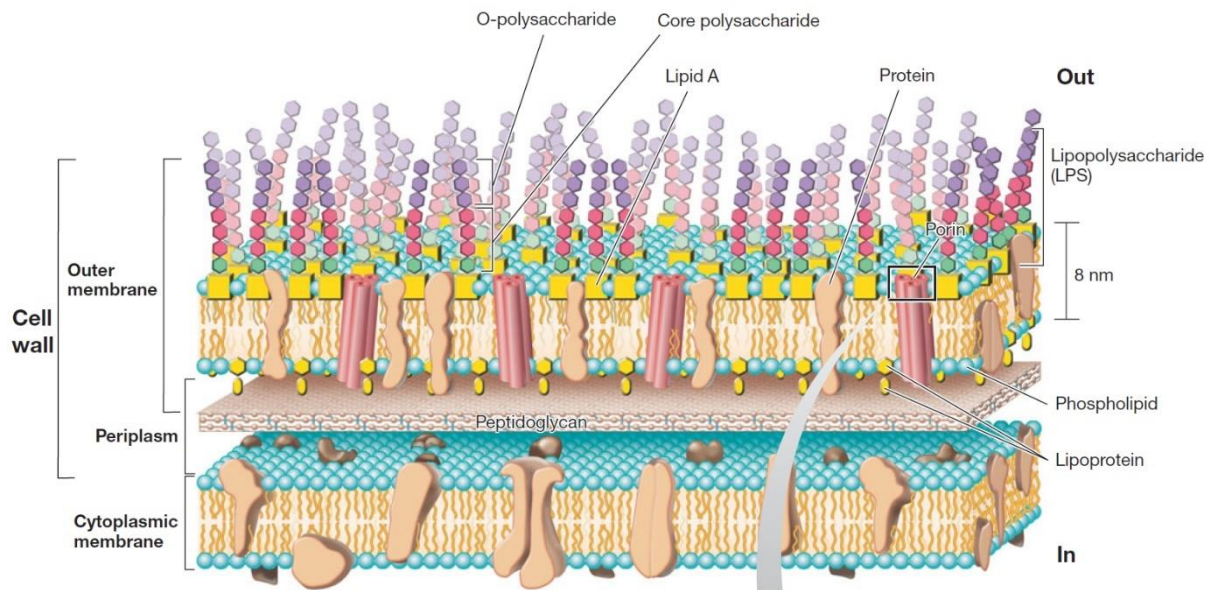


Figure 1. Gram negative bacteria cell envelope (image from Biology of Microorganism, Brock, 10th edition [M. Madigan, 2017])

The inner membrane is a phospholipid bilayer. In *E. coli* this membrane is composed mainly of phosphatidyl ethanolamine and phosphatidyl glycerol, and to some extent also of phosphatidyl serine and cardiolipin [Raetz and Dowhan, 1990].

Unlike the IM, only the inner leaflet of the outer membrane is composed of phospholipids. The outer leaflet is composed of glycolipids, mainly lipopolysaccharides (LPS), also termed endotoxins [Kamio and Nikaido, 1976]. The innate immune system is sensitized to LPS because it is marker of infection, and its release upon cell lysis can cause the endotoxic shock associated with the septicaemia by Gram-negative organisms [Raetz and Whitfield, 2002]. LPS is critical for the barrier function of the OM. It is composed of lipid A, a glucosamine disaccharide with multiple fatty acid chains anchoring LPS to the bilayer, a polysaccharide core, and an extended polysaccharide chain that is called the O-antigen and may vary within a species between strains [Raetz and Whitfield, 2002]. Pathogenic *E. coli* strains can be classified by the antigenic properties of their O-antigen and the flagellar protein flagellin, termed H, e.g. *E. coli* O157:H7. LPS molecules can bind each other avidly, especially in the presence of Mg²⁺, and the acyl chains are highly saturated, facilitating tight packing. This makes the LPS membrane a very effective nonfluid barrier for hydrophobic molecules. Most proteins of the OM are classified as either lipoproteins or β -barrel proteins. The former are anchored to the lipid bilayer via a lipid moiety attached to an N-terminal cysteine residue [Sankaran and Wu, 1994]. The latter comprise almost all of the integral transmembrane proteins of the OM. These proteins contain large portions folding into β -sheets that are wrapped into cylinders, and are referred to as outer membrane proteins, OMPs. Some of them (e.g. OmpF, OmpC) serve to allow the passive diffusion of small molecules such as monosaccharides, disaccharides and amino-acids. The porins present in the OM limit diffusion of hydrophilic molecules larger than c.a. 700 Daltons. Combined, the LPS and the porins turn the OM into a very effective selective permeability barrier [Nikaido, 2003].

The periplasm, densely packed with proteins, is more viscous than the cytoplasm [Mullineaux *et al.*, 2006]. Cellular compartmentalization allows Gram-negative bacteria to sequester potentially harmful degradative enzymes such as RNAses or alkaline phosphatases. Thus, the periplasm has been called

an evolutionary precursor of the lysosomes of eukaryotes [De Duve and Wattiaux, 1966]. Other proteins reside in this compartment as well, including the periplasmic binding proteins, taking part in sugar and amino acid transport and chemotaxis, and chaperone-like molecules, participating in envelope biogenesis [Ehrmann, 2007].

The cell wall is formed by peptidoglycan, also called murein. Peptidoglycan is made up of repeating units of the disaccharide N-acetylglucosamine (NAG or GlcNAc) and N-acetylmuramic acid (NAM or MurNAc), cross-linked by three to five peptide side chains [Vollmer *et al.*, 2008] (Figure 2). The cross-linking of the peptide chains turns peptidoglycan into a 3D mesh-like layer. The cell wall serves a structural role in the bacterial cell wall, giving structural strength, and resisting the osmotic pressure of the cytoplasm.

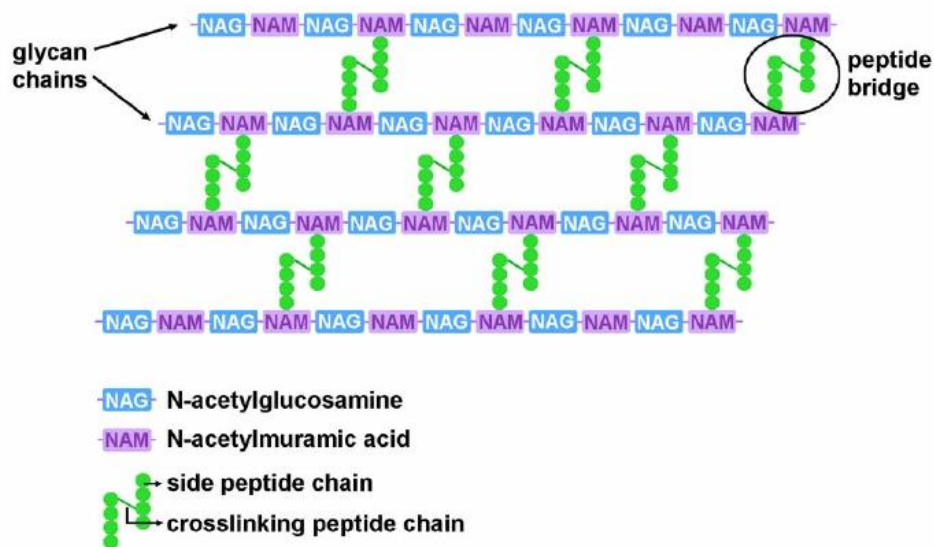


Figure2. Representation of the peptidoglycan structure (Peptidoglycan hydrolases as novel tool for anti-enterococcal therapy, Maliničová).

The peptidoglycan monomers are synthesized inside the cell, attached to a membrane carrier, bactoprenol, and transported across the IM. Once in the periplasm, they are inserted into the existing peptidoglycan by transglycosylation. The C4 hydroxyl group of the GlcNAc will attach to the C1 of MurNAc in the glycan, displacing the lipid-PP from the glycan chain. The enzymes responsible for this are transglycosidases [White *et al.*, 2011].

C) Penicillin Binding Proteins:

The D,D-transpeptidases have been historically referred to as penicillin-binding proteins (PBPs) since these enzymes were routinely identified by covalent labelling with radioactive penicillin followed by gel electrophoresis [Zapun *et al.*, 2008]. PBPs catalyse the polymerization of the glycan strand (transglycosylation) and the cross-linking between glycan chains (transpeptidation) (Figure 3). These enzymes catalyse the formation of peptidoglycan cross-links in a two-step reaction [Sauvage *et al.*, 2008]. PBPs share a common D,D-peptidase activity, whether a DD-transpeptidase, a DD-carboxypeptidase or a D,D-endopeptidase activity [Ghuysen, 1991; Goffin and Ghuysen, 1998]. The carboxypeptidation and transpeptidation reactions catalysed by PBPs follow a three-step mechanism: the rapid, reversible formation of a non-covalent Henri-Michaelis complex between the enzyme and

a peptidoglycan stem pentapeptide [L-Ala-γ-D-Glu-(A2pm/L-Lys)-D-Ala-D-Ala], called the donor strand, is followed by the attack of the active serine on the carbonyl carbon atom of the C-terminal D-Ala-D-Ala peptide bond, leading to the formation of an acyl-enzyme intermediate and the concomitant release of the C-terminal D-Ala (acylation). The final step (deacylation) consists of either hydrolysis, with release of the shortened peptide (carboxypeptidation), or cross-link formation with a second peptidoglycan stem peptide called the acceptor strand (transpeptidation). The D,D-endopeptidase activity of PBPs consists of the hydrolysis of the cross-bridge resulting from the DD-transpeptidase activity [Sauvage *et al.*, 2008].

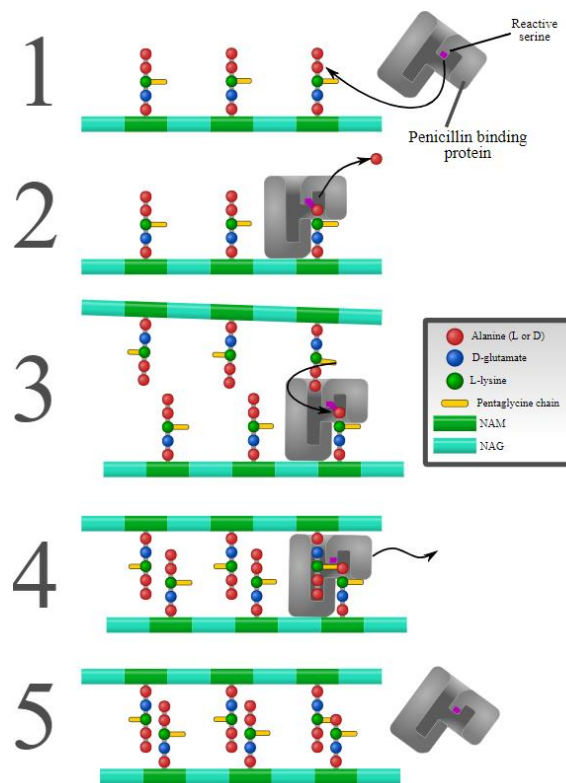


Figure 3. Representation of the cross-link formation. (1) The bacterial cell wall consists of strands of repeating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) subunits. The NAM subunits have short peptide chains attached to them. These chains are bound to chains of 5 glycine residues that will be used in cross-linking. (2) The PBP forms a bond with the peptide side chain at the second most distal alanine residue. This displaces the most distal alanine residue. (3) Another strand of bacterial cell wall arrives. The free end of one of the pentaglycine chains displaces the PBP and forms a bond with the terminal alanine on the other strand. (4) After being displaced, the PBP diffuses away. (5) The formation of one cross-link is complete.

PBPs have been divided into two main categories, the high molecular mass (HMM) PBPs and the low molecular mass (LMM) PBPs. HMM PBPs are multi-modular PBPs responsible for peptidoglycan polymerization and insertion into pre-existing cell wall [Born *et al.*, 2006; Goffin and Ghuysen, 1998]. Depending on the structure and the catalytic activity of their N-terminal domain, they belong either to class A or class B PBPs. The C-terminal penicillin binding (PB) domain of both classes has a transpeptidase activity, catalysing peptide cross-linking between two adjacent glycan chains. In class A, the N-terminal domain is responsible for their glycosyltransferase activity, catalysing the elongation of uncross-linked glycan chains. In class B, the N-terminal domain is believed to play a role in cell

morphogenesis by interacting with other proteins involved in the cell cycle [Höltje, 1998; Den Blaauwen *et al.*, 2008; Zapun *et al.*, 2008]. Monofunctional enzymes (MGTs) similar to the glycosyltransferase domain of class A PBPs also exist in some bacteria but their exact role is still unknown [Spratt *et al.*, 1996].

Because of the structural resemblance between their natural substrate, the D-Ala-D-Ala end of the stem pentapeptide precursors, and penicillin, the late stage peptidoglycan synthesizing enzymes are sensitive to penicillin with which they form a long-lived acyl-enzyme complex that impairs their peptidoglycan cross-linking capability [Tipper and Strominger, 1965].

β-lactams antibiotics:

A) mode of action:

β-lactam antibiotics act by inhibiting the PBPs and consequently the bacterial wall synthesis. They mimic the substrate of PBPs, the D-Ala-D-Ala peptide (Figure 4).

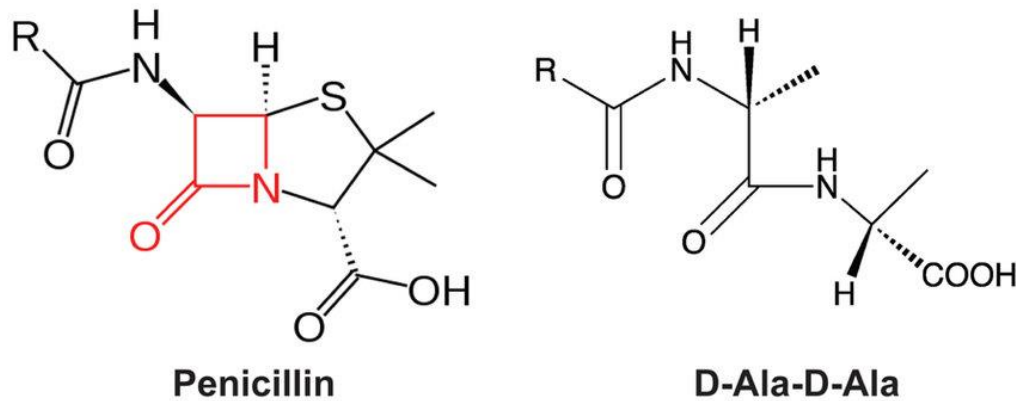


Figure 4. β-lactams mimic the peptide substrate of PBPs

Just as with the dipeptide, the PBP performs a nucleophilic attack on the β-lactam ring with the active site serine (Figure 5). Unlike with the peptide substrate, however, the acyl-enzyme complex between β-lactams and PBPs is stable and not readily hydrolysed, thus rendering the enzyme inactive and inhibiting the transpeptidation of the peptidoglycan [Golemi-Kotra *et al.*, 2004]. In addition, the antibiotic-PBP complex supposedly stimulates the release of autolysins, enzymes that digest the existing cell wall [M. Madigan, 2017]. As a consequence, the bacterial cell wall is weakened, and the osmotic pressure differences between the inside and outside of the cell cause lysis [Park and Strominger, 1957]. β-lactam antibiotics target a vital cellular function of bacteria not present in the host cells. Their effectiveness and generally low toxicity make them one of the most important groups of antibiotics. Together, the β-lactam antibiotics account for over one-half of all of the antibiotics produced and used worldwide.

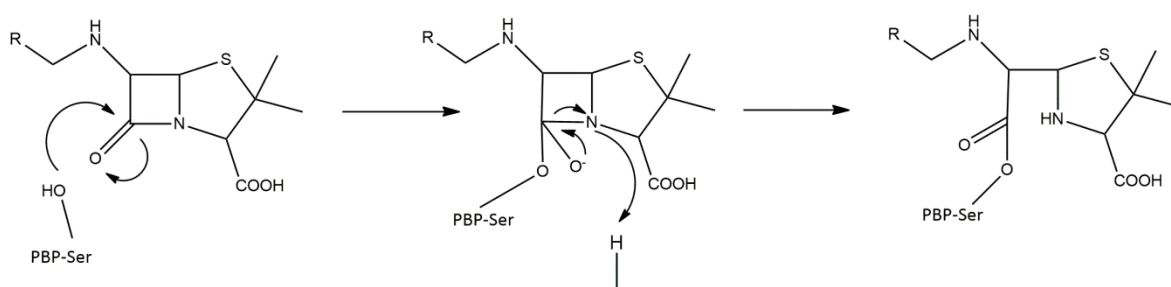


Figure 5. Mechanism of action of β -lactams. The hydroxyl of the serine of the PBP attacks the amide bond and forms a tetrahedral intermediate. The tetrahedral intermediate collapses, the amide bond is broken and the nitrogen is reduced. The PBP is now covalently bound to the drug and cannot anymore perform the cross linking reaction.

B) Structure and spectrum of activity:

The common structural feature of these antibiotics is the highly reactive four-membered β -lactam ring. The clinical success of the first β -lactam, penicillin G (benzylpenicillin), prompted the search for and development of additional derivatives. This quest gave rise to the β -lactam antibiotic families in clinical use today: penicillins, narrow- and extended-spectrum cephalosporins, monobactams, and carbapenems [Babic *et al.*, 2006] (Figure 6).

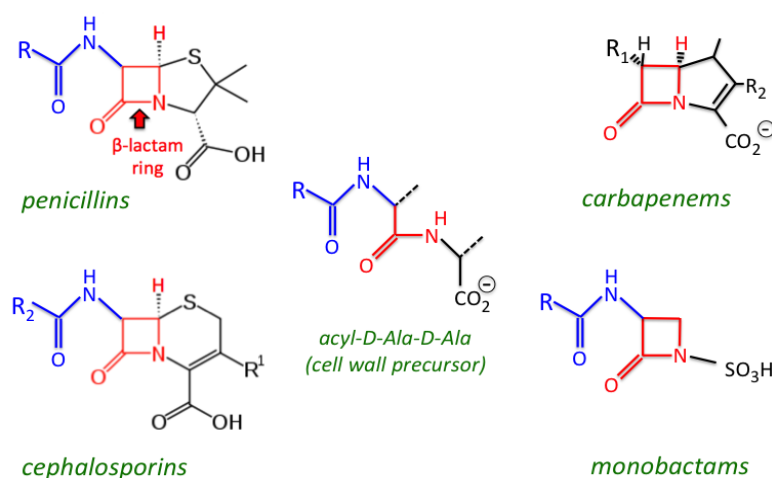


Figure 6. Core structure of different β -lactam families.

C) History and classification:

a. Penicillins

Belonging to penams, penicillins possess a bicyclic structure, 6-aminopenicillanic acid or 6-APA (Figure 7a). This structure is composed of an enclosed dipeptide formed by the condensation of L-cystein and D-valine, resulting in the β -lactam ring and in the thiazolidine ring [Rojas *et al.*, 2016]. Penicillin G (Figure 7b) (benzylpenicillin) was the first β -lactam to be used clinically, most frequently to treat streptococcal infections for which it had high potency [Rammelkamp and Keefer, 1943; Hirsh and

Dowling, 1946]. Another naturally occurring penicillin, penicillin V (phenoxymethylpenicillin, Figure 7c), in an oral formulation is still used therapeutically and prophylactically for mild to moderate infections caused by susceptible *Streptococcus* spp., including use in paediatric patients [Pottegård *et al.*, 2015].

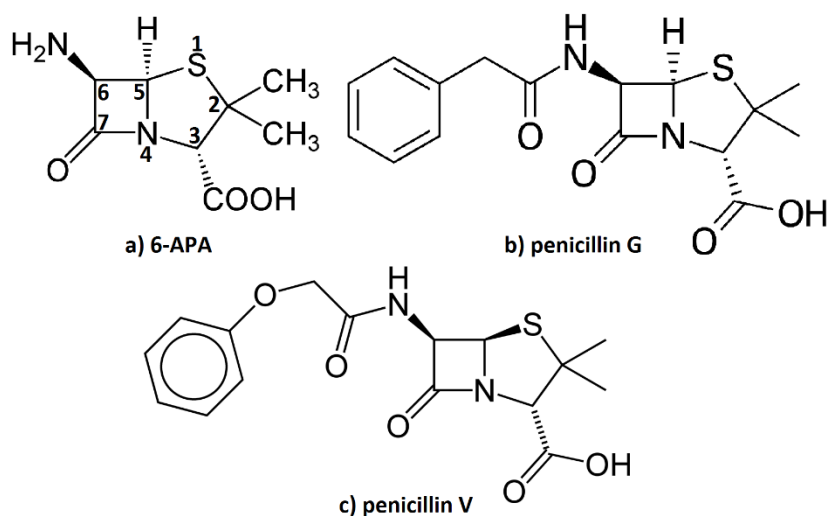


Figure 7. 6-APA and examples of penicillin structures.

Among the penicillinase-stable penicillins of clinical significance are methicillin, oxacillin, cloxacillin, and nafcillin (Figures 8a,b,c,d), with the latter suggested as the β -lactam of choice for skin infections, catheter infections, and bacteraemia caused by methicillin-susceptible *S. aureus* [Bamberger and Boyd, 2005]. Penicillins with improved activity against Gram-negative pathogens included the orally bioavailable ampicillin and amoxicillin (Figures 9a,b), both of which were introduced in the 1970s.

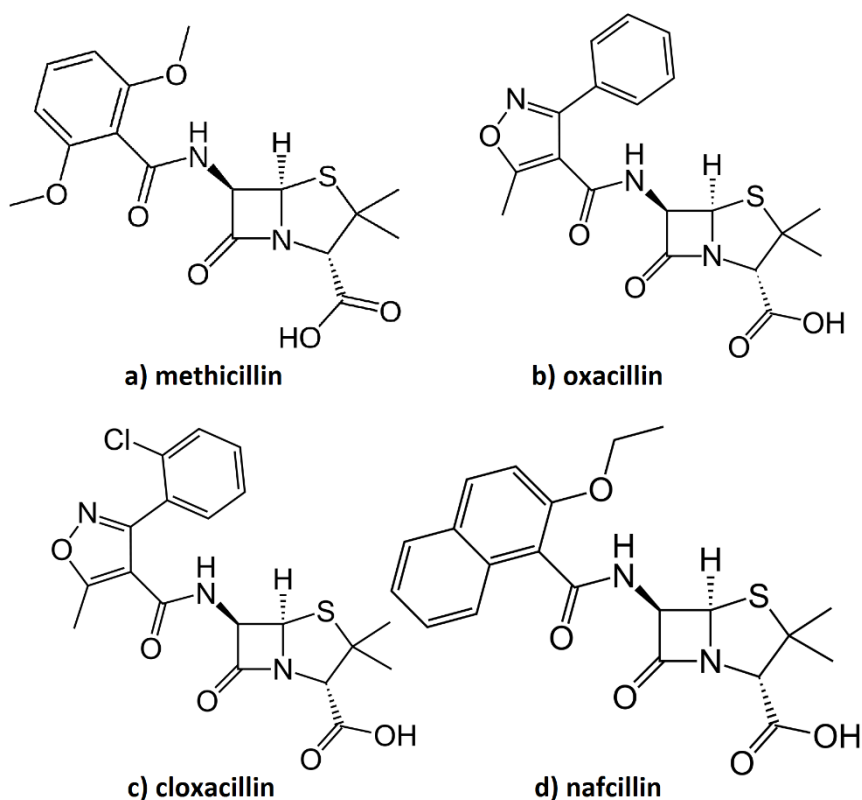


Figure 8. Examples of penicillinase-stable penicillin structures.

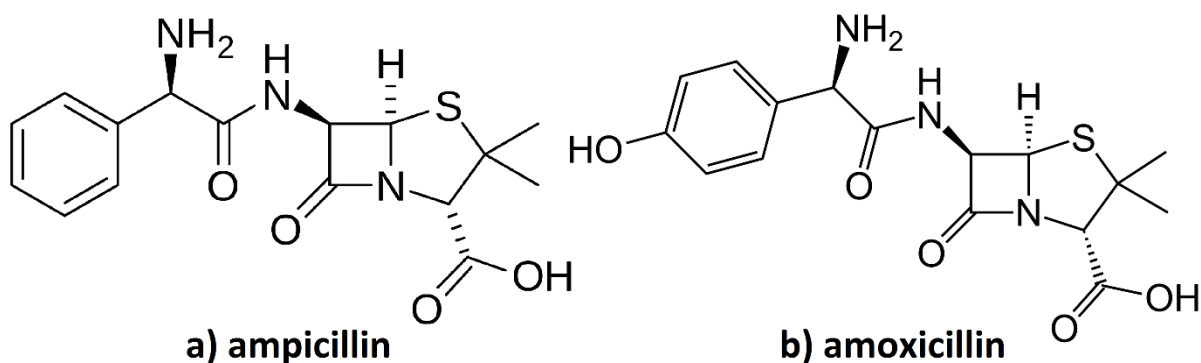


Figure 9. Examples of penicillin structures with improved activity against Gram-negative pathogens.

Carbenicillin (Figure 10a) was the first antipseudomonal penicillin to be introduced, but lacked stability to β -lactamase hydrolysis and was less potent than piperacillin or ticarcillin (Figures 10b,c), later antipseudomonal penicillins. These latter drugs were considered to be potent broad-spectrum penicillins that included penicillin-susceptible staphylococci, enteric bacteria, anaerobes, and *P. aeruginosa* in their spectrum of activity.

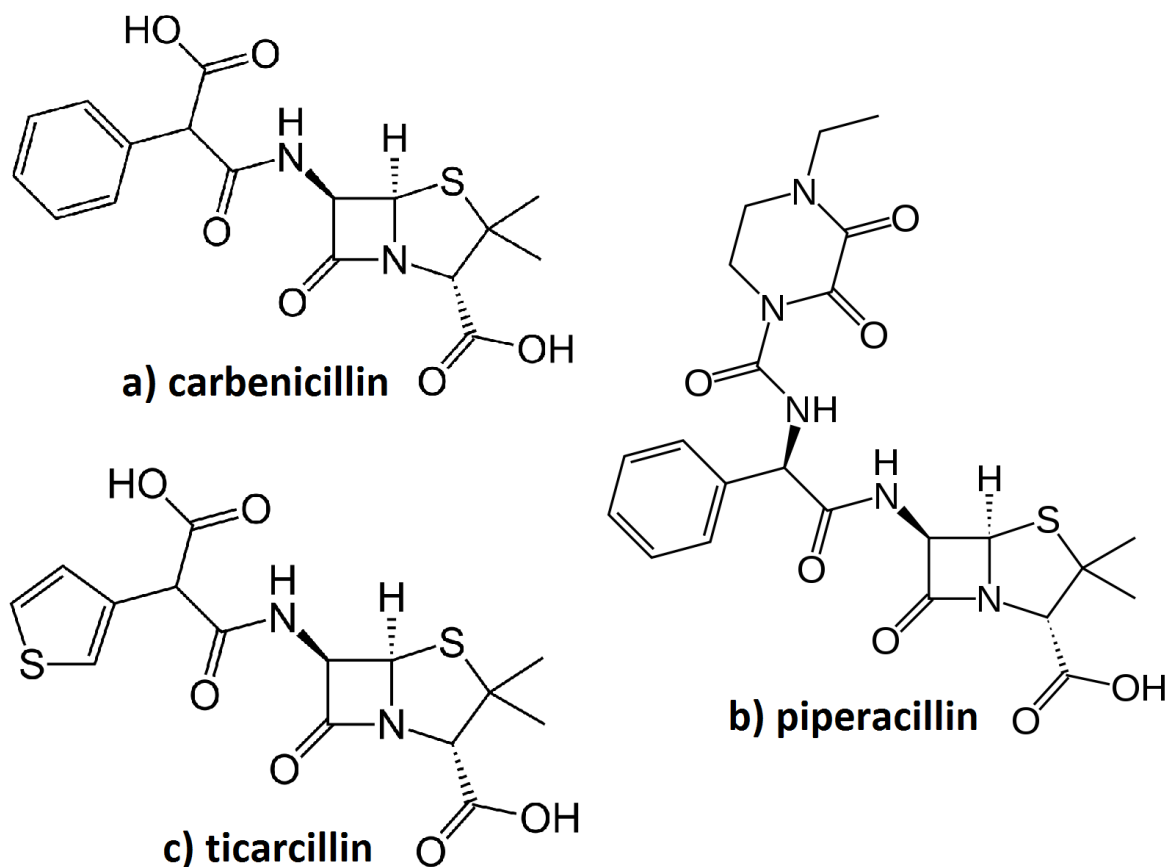


Figure 10. Examples of antipseudomonal penicillin structures.

Two parenteral penicillins with unusual chemical structures, mecillinam and temocillin (Figures 11a,b), were introduced in the late 1980s. Mecillinam (also known as amdinocillin), with a 6- β -amidino side chain, is a narrow-spectrum β -lactam that binds exclusively to PBP2 in enteric bacteria [Curtis *et al.*, 1979]. Because of this specificity, it shows synergy *in vitro* in combination with other β -lactams that bind to PBPs 1a/1b and/or PBP3 in Gram-negative bacteria [Hanberger *et al.*, 1991], thus decreasing

the possibility that a point mutation in a single PBP would lead to resistance [Hickman *et al.*, 2014]. Temocillin, the 6- α -methoxypenicillin analogue of ticarcillin, had greater stability than ticarcillin to hydrolysis by serine β -lactamases, but lost antibacterial activity against Gram-positive bacteria, anaerobic Gram-negative pathogens, and some enteric bacteria that included the important pathogens *Enterobacter* spp. and *Serratia marcescens* [Martinez-Beltran *et al.*, 1985].

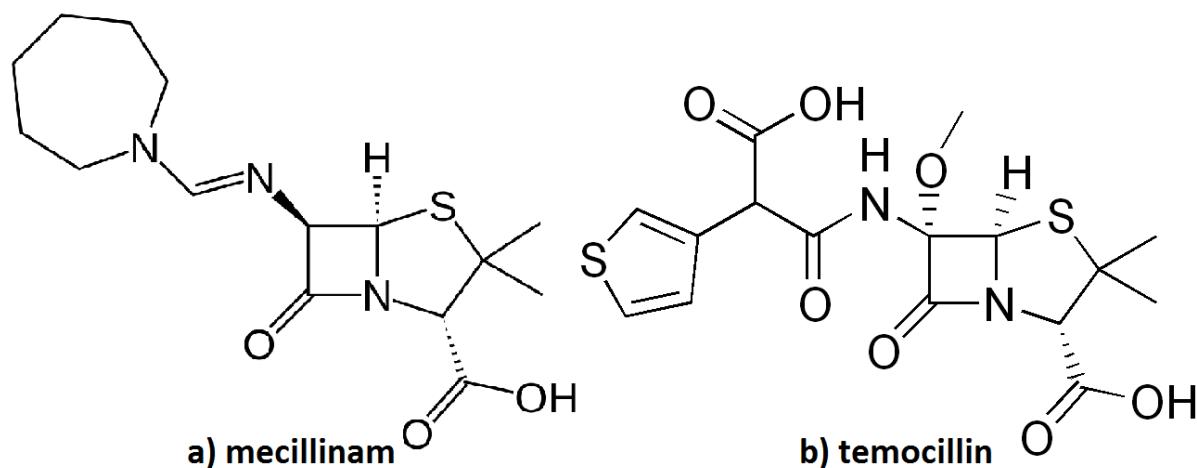


Figure 11. Examples of antipseudomonal penicillin structures.

b. Cephalosporins

Since 1970s, cephalosporins, the major representative group of cephems, have been among the most potent and most widely used anti-infective agents. The original *Cephalosporium acremonium* culture was discovered in a sewer outlet in Sardinia (1954) [Newton and Abraham, 1955] from which the cephalosporin C (Figure 12b) was isolated, a weak antibiotic compound that showed some activity against penicillin-resistant cultures. Chemical removal of the cephalosporin C side-chain forms 7-aminocephalosporanic acid or 7-ACA (Figure 12a), which, like its congener 6-APA, was used as a synthetic starting point for most of the cephalosporins available today. It is now more economically feasible to produce 7-ACA from penicillin G in a seven-step synthesis, rather than to incur the cost of large-scale fermentation of cephalosporin C [Velasco *et al.*, 2000]. Because of their importance, it is essential to classify cephems in order to allow their optimal use. Numerous classifications have been proposed: chemical, biological, microbiological, pharmacokinetic and immunological. Here we describe the microbiological classification.

- Microbiological classification

Cephalosporins are divided into first-generation, second-generation, third-generation, fourth generation and fifth-generation, according to their antibacterial activity.

First-generation cephalosporins are very active against Gram-positive cocci, except enterococci and methicillin-resistant staphylococci, and moderately active against some Gram-negative rods primarily *Escherichia coli*, *Proteus*, and *Klebsiella*. Anaerobic cocci are often sensitive, but *Bacteroides fragilis* is not. Cephalixin and cephalotin (Figures 12c,d) belong to this group.

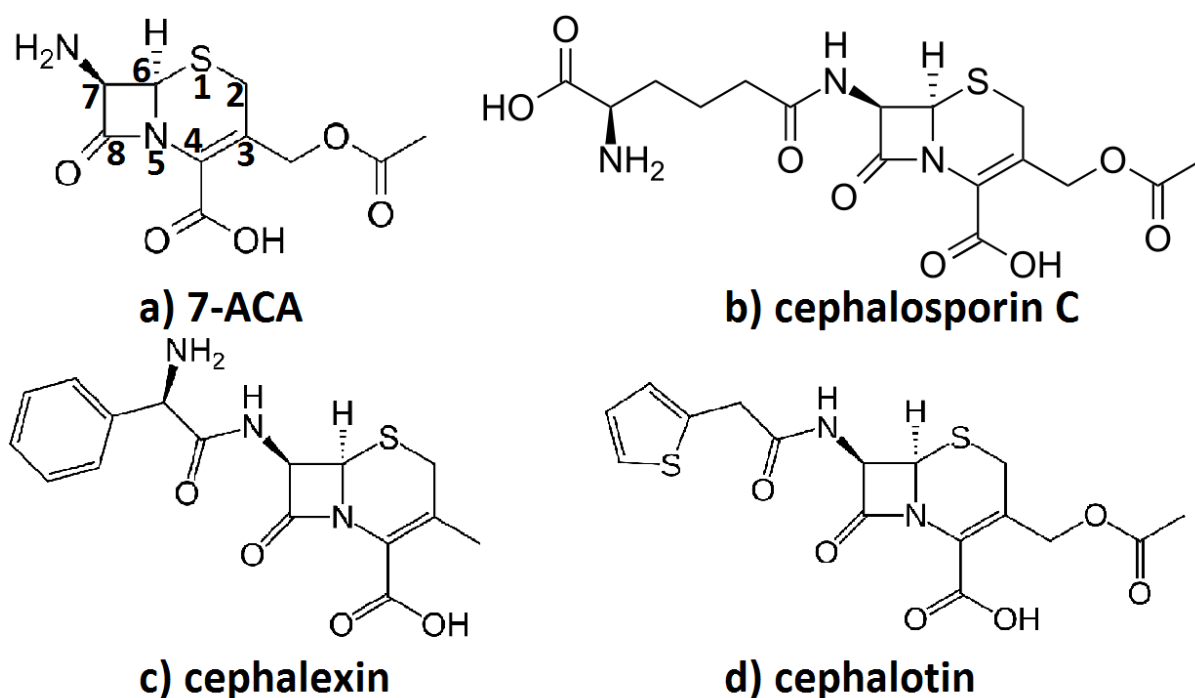


Figure 12. 7-ACA and examples of first-generation cephalosporin structures.

Second-generation cephalosporins are active against organisms covered by first-generation drugs, but have extended coverage against Gram-negative rods, including *Klebsiella* and *Proteus*, but not against *Pseudomonas aeruginosa* [Bennett *et al.*, 2012]. Some examples are cefoxitin and cefuroxime (Figures 13a,b).

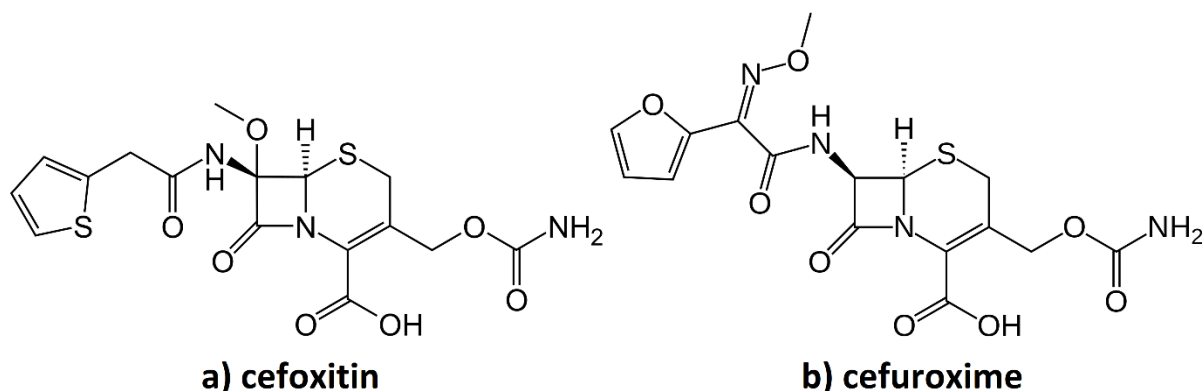


Figure 13. Examples of second-generation cephalosporin structures.

Third-generation cephalosporins enhanced activity against Gram-negative rods [Paladino *et al.*, 2008]. Whereas second-generation drugs tend to fail against *P. aeruginosa*, ceftazidime or cefoperazone (Figures 14a,d) may succeed. Thus, third-generation drugs are very useful in the management of hospital-acquired Gram-negative bacteraemia. Another important distinguishing feature of several third generation drugs is the ability to reach the central nervous system and to appear in the spinal fluid in sufficient concentrations to treat meningitis caused by Gram-negative rods [O'Neill *et al.*, 2006]. Cefotaxime, ceftriaxone or ceftizoxime (Figures 14b,c,e) are few examples of this group.

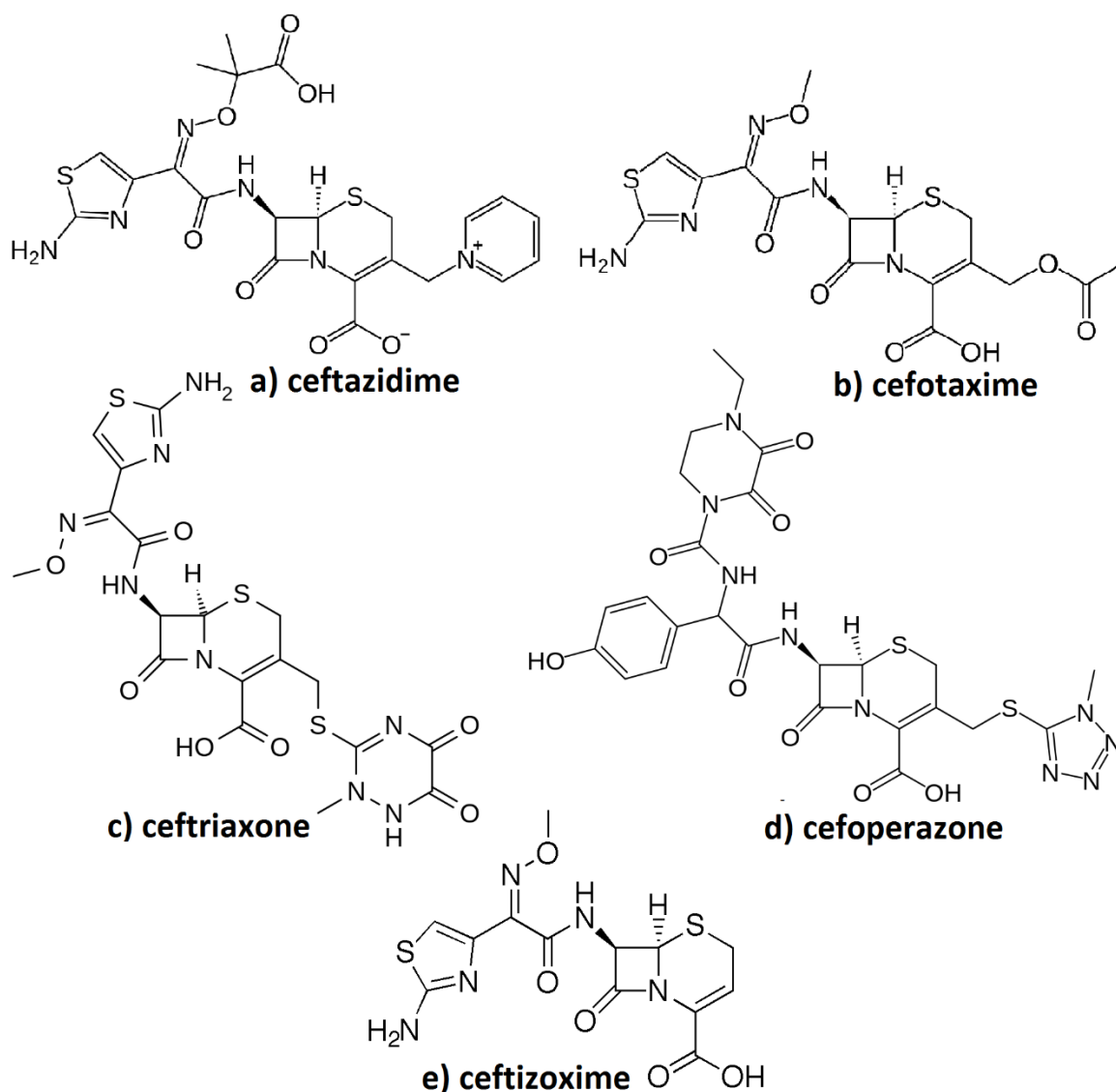


Figure 14. Examples of third-generation cephalosporin structures.

Fourth-generation cephalosporins have enhanced activity against *Enterobacter* and *Citrobacter* species that are resistant to third-generation cephalosporins. Cefepime (Figure 15a) has activity comparable with that of ceftazidime against *P. aeruginosa*. The activity against streptococci and methicillin-susceptible staphylococci is greater than that of ceftazidime and comparable with that of the other third-generation compounds [Fritsche *et al.*, 2008].

Fifth-generation cephalosporins were developed in the laboratory to specifically target against resistant strains of bacteria. Particularly, ceftobiprole (Figure 15b) is effective against methicillin-resistant *S. aureus* (MRSA). Ceftaroline (Figure 15c) is a new oxyimino-cephalosporin that is also effective against MRSA [Gould *et al.*, 2012], but ineffective against extended-spectrum β -lactamase (ESBL) producers or active AmpCs. However, ceftaroline has showed to be effective against broader spectrum β -lactamases, in synergism with amikacin [Vidaillac *et al.*, 2009].

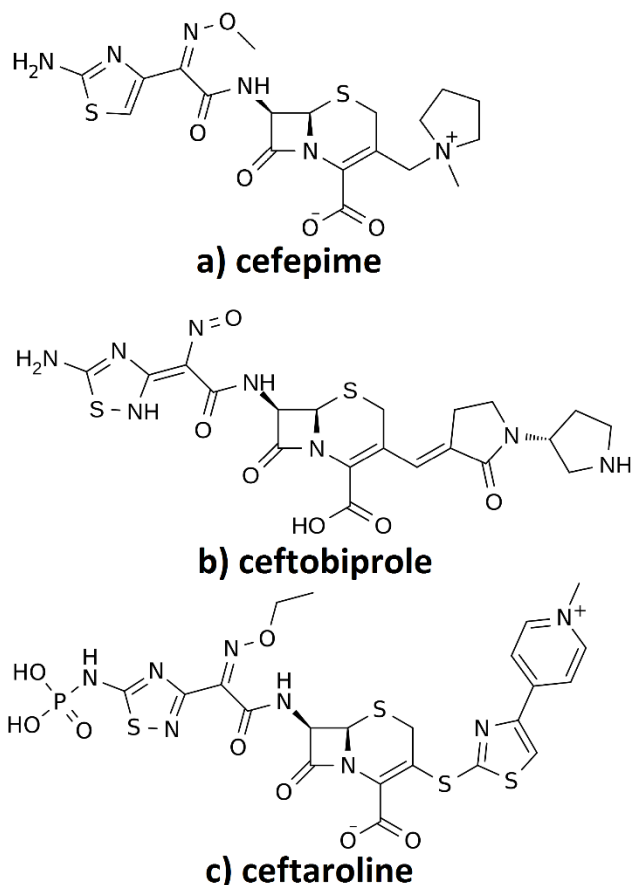


Figure 15. Examples of fourth- and fifth-generation cephalosporin structures.

c. Carbapenems

Thienamycin (Figure 16a) was identified in the mid-1970s as a potent broad-spectrum antibiotic with the typical four-membered β -lactam structure fused to a novel five-membered ring in which carbon rather than sulfur was present at the 1-position [Kahan *et al.*, 1979]. Because of its chemical instability, this carbapenem was never developed as a therapeutic agent, but was stabilized by adding the N-formimidoyl group to the 2-position, resulting in imipenem (Figure 16b). Carbapenems, in general, bind strongly to PBP2 in Gram-negative bacteria, but may also bind to PBP1a, 1b, and 3, thus providing supplemental killing mechanisms that may serve to lessen the emergence of resistance [Sumita and Fukasawa, 1995; Yang *et al.*, 1995]. Because of the lability of imipenem to hydrolysis by the human renal dehydropeptidase (DHP) causing inactivation of the drug [Kropp *et al.*, 1982], it is dosed in combination with cilastatin, a DHP inhibitor that also acts as a nephroprotectant [Kahan *et al.*, 1983]. Following the introduction of imipenem, later carbapenems containing a 1 β -methyl group that conferred stability to the human DHP have been developed [Zhanel *et al.*, 2007]. These are meropenem, ertapenem and doripenem (Figures 16c,d,e), among others, with generally the same group of organisms included in their potent spectrum of activity [Baughman, 2009].

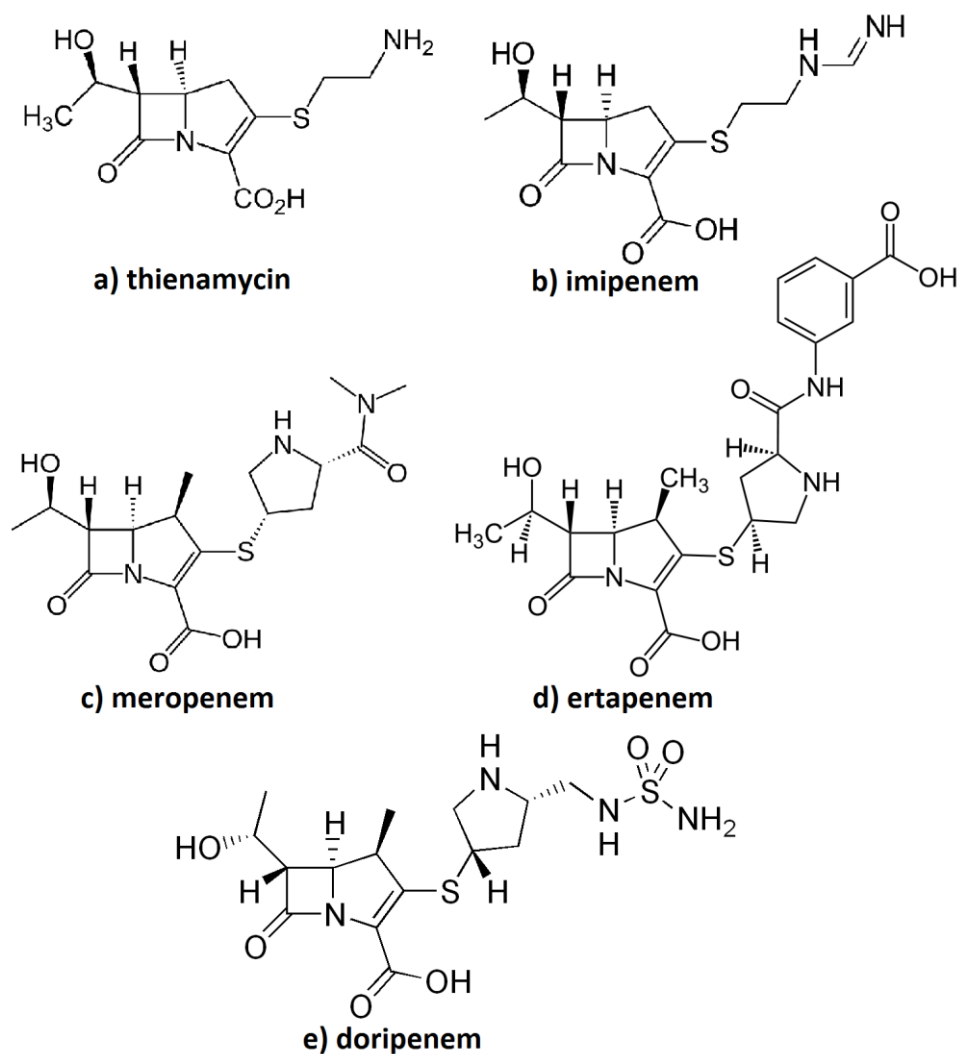


Figure 16. Examples of carbapenem structures.

d. Monobactams

Aztreonam (Figure 17) is the most representative monobactam. It is a monocyclic β -lactam with an N1-sulfonic acid substituent, originated as a derivative from a novel antibiotic isolated from the New Jersey Pine Barrens [Georgopapadakou *et al.*, 1983]. It binds tightly to PBP3 in Gram-negative rods, with weaker binding to PBP1a, leading to filamentation followed by cell lysis [Sykes *et al.*, 1982]. They are active against Gram-negative rods, but not against Gram-positive bacteria or anaerobes [Bennett *et al.*, 2012].

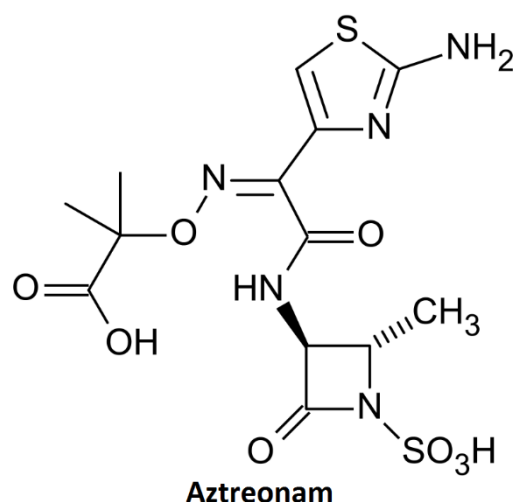


Figure 17. Examples of a monobactam structure.

D) Mechanism of resistance to β -lactams:

Four basic mechanisms may contribute to β -lactam resistance in bacteria (Figure 18).

a. Impermeability

Impermeability, which is the result of a reduction of the amount of a given antimicrobial that reaches the target, is related to the size and physicochemical properties of that compound. The acquired resistance due to reduction of the cell wall permeability has been reported in *E. coli*, *Proteus sp.*, *Salmonella sp.*, *Shigella sp.*, *Klebsiella sp.*, *Enterobacter sp.* and *Serratia sp.*, either through quantitative or qualitative alteration of the porins. This mechanism generally confers reduced susceptibility to antibiotics and is often accompanied with β -lactamase expression. In Enterobacteriaceae this mechanism is not specific to β -lactams and can affect other antimicrobial families as well [Martínez-Martínez, 2008].

b. Efflux systems

Efflux pumps are membrane active transporters that excrete toxic molecules outside the cytoplasm. These efflux pumps can be classified according to their structure, the type of molecule that are extruded, or the resistance phenotype they confer. There are five families of efflux pumps associated to the resistance, ABC (ATP-Binding Cassette transporters), SMR (Small Multidrug Resistance), MATE (Multidrug and Toxic Compound Extrusion), MFS (Major Facilitator Superfamily) and RND (Resistance Nodulation cell Division) [Li and Nikaido, 2009]. They also can be differentiated according to the source of energy used for the molecular transport, the proton motive force (MFS, RND and SMR family), sodium ions (MATE family) or the hydrolysis of adenosine triphosphate (ABC transporters) [Cattoir, 2004].

c. Alteration of the target site

Because of the vital cellular functions of the target enzymes (PBPs), bacteria cannot evade antimicrobial action by inactivating them entirely. However, it is possible for the target to acquire mutations that reduce their susceptibility to inhibition whilst retaining cellular-function. Mutations that achieve this occur in RNA polymerase and DNA gyrase, resulting in resistance to the rifampicin and quinolones, respectively. In some cases, the modification in target structure needed to produce resistance (interfering with binding of the antimicrobial agent) requires other changes in the cell to compensate for the altered characteristics of the target [Lambert, 2005]. This is the case in the most

important example of a target change, the acquisition of the altered transpeptidase MecA in *Staphylococcus aureus* that results in resistance to methicillin (methicillin-resistant *S. aureus*, MRSA) and to most other β -lactam antibiotics. To continue to function efficiently in peptidoglycan biosynthesis, MecA needs alterations to be made in the composition and structure of the peptidoglycan, which involves functioning of a number of additional *mecA* gene [Enright *et al.*, 2002]. Other important example is the altered PBPs in *Neisseria gonorrhoeae*. *N. gonorrhoeae* has four PBPs, designated PBP 1, 2, 3, and 4 [Barbour, 1981]. Of these, only PBP 1 and 2 are essential for cell viability and are, thus, potential antibiotic killing targets in *N. gonorrhoeae*. Because penicillin G has an approximately 10-fold higher rate of acylation of PBP 2 than of PBP 1, it kills *N. gonorrhoeae* at its MIC by inactivation of PBP 2. Penicillin-resistant strains of *N. gonorrhoeae* are capable of transferring their resistance genes to susceptible strains via transformation and homologous recombination. Susceptible strains of *N. gonorrhoeae* become resistant to penicillin by acquiring multiple resistance genes in a stepwise fashion. Transformation to high-level penicillin resistance is mediated by the *penA* gene, encoding altered forms of PBP 2 that display 5- to 10-fold decreases in their rate of acylation by penicillin [Spratt, 1988]. Mutations in PBP 1 (encoded by the *ponA* gene) can also contribute to high-level penicillin resistance. A mutation in *ponA* producing a single amino acid change in PBP 1 has been identified in penicillin-resistant *N. gonorrhoeae* strains [Ropp *et al.*, 2002]. In *Neisseria meningitidis* mosaic PBPs were observed, conferring resistance. The PBP 2 genes (*penA*) of penicillin-resistant *N. meningitidis* have a mosaic structure generated by the introduction of segments of the *penA* genes of related *Neisseria* species [Bowler *et al.*, 1994] particularly the commensal species *Neisseria flavescens* and *Neisseria cinerea* [Spratt *et al.*, 1989, 1992].

d. Enzymatic inactivation

The most important β -lactam resistance mechanism in bacteria, particularly Gram-negative bacteria, is the hydrolysis by β -lactamases. They inactivate these antibiotics by opening the β -lactam ring. Some species of Enterobacteriaceae naturally possess chromosomal β -lactamase genes that make them resistant to aminopenicillins and first-generation cephalosporins. These are expressed constitutively in some cases (e.g. *K. pneumoniae* SHV penicillinase), and induced by regulatory genes in other cases (e.g. *Serratia sp.*, *Enterobacter sp.*, *Morganella sp.*, *Providencia sp.* cephalosporinase).

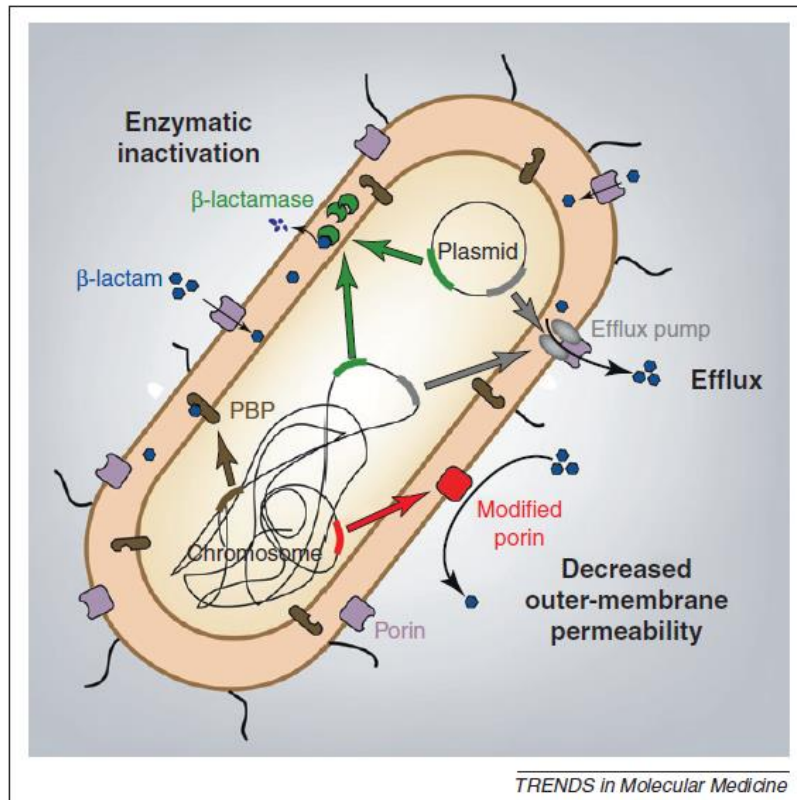


Figure 18. Mechanisms of β -lactam resistance in *Enterobacteriaceae*. Primary mechanisms of β -lactam resistance in *Enterobacteriaceae* include the following: (i) enzymatic inactivation of the antibiotic by β -lactamases; (ii) decreased outer membrane permeability through production of modified porins, loss of porin expression, or a shift in the types of porins found in the outer membrane; and (iii) efflux of the antibiotic to the outside of the bacterium through production of an efflux pump. Image from [Nordmann *et al.*, 2012].

β -lactamases:

A) Classification:

a. Ambler classification:

β -lactamases can be classified in four classes according to their amino-acid sequence identity [Ambler, 1980]. Classes A, C, and D include enzymes that hydrolyse their substrates by forming an acyl-enzyme through an active site serine, whereas class B β -lactamases are metalloenzymes that utilize at least one active-site zinc ion to facilitate β -lactam hydrolysis (Table 1)

Class A β -lactamases: they represent the most important group. They can hydrolyse penicillins (amoxicillin, ticarcillin, piperacillin) and are susceptible to β -lactamases inhibitors (clavulanic acid, tazobactam and sulbactam).

Class B β -lactamases: they are metalloproteins that possess at least one zinc ion in the active site. These β -lactamases are resistant to the classic inhibitors but can be inhibited by divalent ion chelators as EDTA (ethylenediaminetetraacetic acid), and also, they cannot hydrolyse aztreonam.

Class C β -lactamases or cephalosporinases: hydrolyse aminopenicillins and cephalosporins (first, second and third generation), are not inhibited by clavulanic acid but are inhibited by cloxacillin.

Class D β -lactamases or oxacillinases: constitute a very heterogeneous group taking into account the hydrolysis profile. Initially, were characterized by the rapid hydrolysis of oxacillin rather than benzylpenicillins, but definition has evolved thanks to the variety of enzymes belonging to this class.

b. Bush and Jacoby classification:

The successive versions of the functional classification published in 1989 [Bush, 1989] and 1995 [Bush *et al.*, 1995] were updated in 2010 [Bush and Jacoby, 2010]. This updated classification considers the hydrolysis profile of the enzyme as well as the inhibition profile, and major groupings are generally correlated with the more broadly-based molecular classification.

This classification presents 3 subgroups (Table 1):

The group 1 includes cephalosporinases of Ambler class C.

The group 2 re-groups the Ambler classes A and D. It includes penicillinases, cephalosporinases, and broad-spectrum β -lactamases that are generally inhibited by active site-directed β -lactamase inhibitors. It is sub-divided in sub-groups that go from 2a to 2f.

The group 3 corresponds to the Ambler class B of classification.

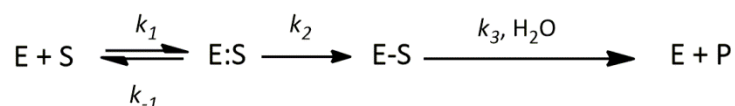
Table 1. Bush and Jacoby β -lactamases classification

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (Ambler)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzymes
				CLA or TZB	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	<i>E. coli</i> AmpC, ACT-1, CMY-2
1e	Not included	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino- β -lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillins than cephalosporins	PC1
2b	2b	A	Penicillins and cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillins and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino- β -lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam and tazobactam	TEM-30, SHV-10
2ber	Not included	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino- β -lactams combined with resistance to clavulanic acid, sulbactam and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	Not included	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime and ceftazidime	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	Not included	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino- β -lactams	OXA-11, OXA-15
2df	Not included	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino- β -lactams. Cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, IND-1
		B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
Not included	4	Unknown					

B) Mechanism of action:

a. Serine β -lactamases

Serine β -lactamases acylate β -lactam antibiotics, much like PBPs, and then use strategically positioned water molecule to hydrolyse the acylated β -lactam [Minasov *et al.*, 2002]. In this manner, the β -lactamase is regenerated and can inactivate additional β -lactam molecules. This enzymatic reaction may be represented by the following equation:



In this scheme, E is a β -lactamase, S is a β -lactam substrate, E:S is the Michaelis-Menten complex, E-S is the acyl-enzyme, and P is the product devoid of antibacterial activity. The rate constants for each step are represented by k_1 , k_{-1} , k_2 , and k_3 . k_1 and k_{-1} are the association and dissociation rate constants for the pre-acylation complex, respectively; k_2 is the acylation rate constant, and k_3 is the deacylation rate constant. In order to be uniform, we now define basic terms that are often used to describe the kinetic behaviour of β -lactamases:

The Michaelis-Menten phenotypically described constant, K_m , is defined as [Galleni and Frère, 2007]

$$K_m = k_3 K_s / (k_2 + k_3)$$

where the thermodynamic constant, K_s , is $(k_{-1} + k_2)/k_1$. The maximum turnover number, k_{cat} , is a composite rate constant that represents multiple chemical steps and is defined as [Copeland, 2005]

$$k_{cat} = k_2 k_3 / (k_2 + k_3)$$

or

$$V_{max} = k_{cat} [E_T]$$

K_m , expressed in terms of concentration, represents the relative affinity of the ES encounter and the rate at which the ES is converted to P; a large K_m value represents poor affinity (large K_s).

After formation of the Michaelis-Menten complex, the active-site serine performs a nucleophilic attack on the carbonyl of the β -lactam antibiotic that results in a high-energy tetrahedral acylation intermediate. This intermediate transitions into a lower-energy covalent acyl-enzyme following protonation of the β -lactam nitrogen and cleavage of the C-N bond. Next, an activated water molecule attacks the covalent complex and leads to a high-energy tetrahedral deacylation intermediate. In the end, the hydrolysis of the bond between the β -lactam carbonyl and the oxygen of the nucleophilic serine regenerates the active enzyme and releases the inactive hydrolysed β -lactam. Acylation and deacylation require the activation of the nucleophilic serine and hydrolytic water, respectively (Figure 19).

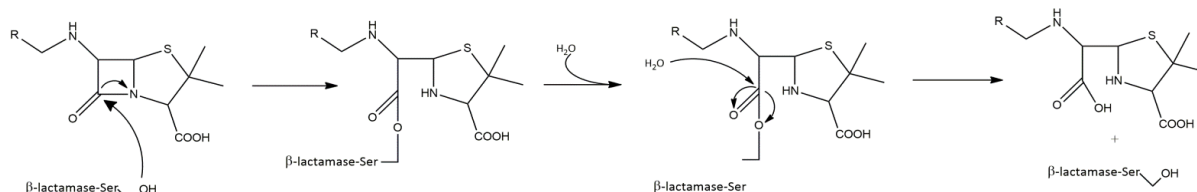


Figure 19. Hydrolytic mechanism of serine β -lactamases

b. Metallo- β -lactamases

In contrast to the serine β -lactamases, class B includes Zn^{2+} -dependent enzymes that follow a different hydrolytic mechanism. In general, MBLs use the HO^- group from a water molecule that is coordinated by Zn^{2+} to hydrolyse the amide bond of a β -lactam (Figure 20). MBLs are divided into three subclasses based on their Zn^{2+} dependency, i.e., whether they (i) are fully active with either one or two ions (subclass B1), (ii) require two ions (subclass B3), or (iii) employ one ion and are inhibited by binding of an additional ion (subclass B2) [Galleni *et al.*, 2001; Hernandez Valladares *et al.*, 1997; Paul-Soto *et al.*, 1999a]. The B1 and B3 subclasses have a broad-spectrum substrate profile that includes penicillins, cephalosporins, and carbapenems while the B2 enzymes exhibit a narrow profile that includes carbapenems [Palzkill, 2013]. MBLs utilizing two Zn^{2+} ions for hydrolysis, such as the subclass B3 *S. maltophilia* L1, coordinate the β -lactam substrate by the carboxylate and carbonyl groups, bridged by a hydroxide ion. After substrate binding, one of the Zn^{2+} ions, in conjunction with enzyme residues, polarizes the β -lactam carbonyl for attack by the HO^- group, which is hydrogen bonded to deprotonated Asp120. Nucleophilic attack by the HO^- group creates a tetrahedral species, which rapidly collapses into an intermediate in which the β -lactam nitrogen is anionic. Protonation of the nitrogen leads to product formation. The source of the proton is not certain, and it may come from Asp120 or a water molecule. The B1 enzyme *Bacillus cereus* BclI is active in both its mononuclear and di-nuclear forms, and in the resting state, the Zn^{2+} -bound HO^- group is hydrogen bonded to the deprotonated Asp120 as well as several other active-site residues [Crowder *et al.*, 2006a; Wang *et al.*, 1999]. After attack by this HO^- group, the breakdown of the tetrahedral intermediate requires protonation of the β -lactam nitrogen. The source of this proton is under investigation. In the case of the CphA B2 MBL from *Aeromonas hydrophila*, a second bound Zn^{2+} ion is inhibitory. The proposed mechanism includes a water molecule activated by either His118 or Asp120, rather than a Zn^{2+} -bound HO^- group. The singular Zn^{2+} appears to help coordinate the β -lactam nitrogen [Garau *et al.*, 2005; Xu *et al.*, 2006].

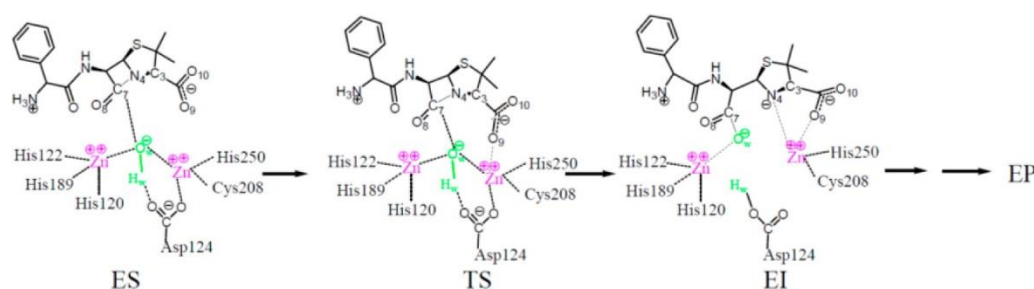


Figure 20. Proposed mechanism for the initial β -lactam ring opening of the NDM-1-catalyzed hydrolysis of ampicillin (Image from [Zheng and Xu, 2013])

c) Serine- β -lactamase structure:

Serine- β -lactamases of classes A, C and D share a few common characteristics. They possess the same overall fold, consisting of two domains, one α -helical domain and one mixed α/β domain [Naas *et al.*, 2016; Powers, 2016; Docquier and Mangani, 2016]. The active site is located in the crevice between these two domains, and the mechanistically important motif S-X-X-K is common to all of them, although the lysine residue presents a post-translational modification in class D enzymes. They also possess a common K-T-G motif in the middle of the β -strand directly adjacent to the active site cleft, and the residue following this motif forms an oxyanion hole with its backbone nitrogen, together with

the active site serine's backbone nitrogen, that stabilized the transition state structures during acylation and deacylation.

a. Class A β -lactamases:

Class A β -lactamases are monomeric enzymes usually around 265 residues in length, and with molecular weights from 25 to 32 kDa. They present four conserved motifs. The first motif is 70SXXK73 (ABL numbering scheme [Ambler *et al.*, 1991]), and the second element, 130SDN132, is equivalent to the conserved motifs YA/SN and SXV/I from class C and class D β -lactamases, respectively. The third one is represented by Glu166, which acts as a general base for the deacylation of the acyl-enzyme complex, being part of a more general 166EXXXN170 motif that is generally found in the Ω -loop. Finally, the 234KTG236 motif is common to most serine-active β -lactamases.

For class A enzymes, two mechanisms have been proposed. In one instance, deprotonated Lys73 is proposed to act as the catalytic base activating Ser70 for nucleophilic attack on the β -lactam carbonyl carbon [Larkin *et al.*, 2007; Robert and Gouet, 2014] as indicated by several class A β -lactamase structures, where Lys73 is H-bonded to Ser70. In a second proposal, Glu166, located on the Ω -loop, would play the role of the catalytic base in acylation [Naas *et al.*, 1994; Mariotte-Boyer *et al.*, 1996]. Ultrahigh resolution crystal structure of TEM-1 (PDB code 1M40) [Rasmussen *et al.*, 1996] provides evidence in favour of the second proposal where Glu166 activates a conserved water molecule that lies at the H-bond distance from Ser70. On the other hand, consensus exists about the deacylation step where the key residue is proposed to be the conserved Glu166, acting as the activating base towards the hydrolytic water molecule [Robert and Gouet, 2014; Rasmussen *et al.*, 1996; Naas *et al.*, 1994].

b. Class C β -lactamases:

Class C β -lactamases possess the S-X-X-K motif (residues 64-67, which contains the catalytic serine residue) and the Y-X-N (residues 150-152) motif. They are characterized by an elaborate active site hydrogen-bonding network that involves conserved residues that have been implicated in catalysis: Lys67, Tyr150, Asn152, Lys315 [Dubus *et al.*, 1994; Monnaie *et al.*, 1994b; Dubus *et al.*, 1995; Monnaie *et al.*, 1994a; Chen *et al.*, 2009]. In the structures of the AmpC and P99 enzymes, Ser64 lies at the center of the active site with its side chain oxygen atom forming hydrogen bonds to the side chains of Lys67 and Tyr150. Lys67 extends the hydrogen bond network toward the lip of the active site via interactions with the side chain of Asn152, which in turn hydrogen bonds to Gln120, all of which are completely conserved residues in class C enzymes [Lobkovsky *et al.*, 1993]. On the other side of the active site, the side chain OH group of Tyr150 interacts with the side chain of Lys315, belonging to common motif KTG, and this completes the hydrogen bond network that encircles the active site region. Several regions were shown to contribute to recognition of β -lactams and inhibitors as well: the R1 amide site, the C3/C4 carboxylate site, and the oxyanion/hydroxyl hole. The R1 amide refers to the conserved amide functional group present in the R1 side chains of β -lactams. The binding site for this amide is composed of the side chains of Gln120 and Asn152 in the AmpC enzymes. Asn152 is completely conserved and is believed to orient the β -lactam substrate in the active site by hydrogen bonding to the R1 amide group [Dubus *et al.*, 1995]. The C3/C4 carboxylate is also a conserved feature of β -lactams, and this functional group is recognized by Asn346 and Arg349 [Powers and Shoichet, 2002; Drawz *et al.*, 2011a]. The oxyanion hole stabilizes the presumed transition state in catalysis and is formed by the main chain amides of residues 64 and 318. The overall three-dimensional structures of extended-spectrum AmpC (ESAC) enzymes are quite similar to narrow-spectrum class C β -lactamases. However, the structural changes in the ESAC variants generally cause the active sites to

become larger, which allows the larger, bulkier late generation cephalosporins to be better accommodated for binding and/or catalysis.

c. Class D β -lactamases:

Class D β -lactamases share the same tertiary structure of other β -lactamases described as a two domains structure. One globular domain consists of seven packed α -helices while the other contains an antiparallel β -sheet surmounted by two α -helices. The β -sheet is made by six or seven strands, depending on the structure. The active site is located at the interface between the two domains. Despite the high sequence divergence (up to 75%), the secondary elements are maintained in all OXA enzymes. In terms of quaternary structure, OXA-23, OXA-24, OXA-58 and OXA-146 are monomeric, like OXA-1, OXA-45 and OXA-85, while OXA-48 assembles as a homodimer, like OXA-10, OXA-13 and OXA-46. The quaternary structure does not appear to have influence on the catalytic properties of these enzymes. The active site of class D β -lactamases shares features similar to those of class A β -lactamases. However, a striking feature of the class D enzymes is the presence of the active site Ser-X-X-Lys conserved motif where a carbamoylated lysine residue is structurally conserved, being located close to the catalytic serine. A comparison of class A β -lactamases with class D enzymes tells that relevant differences exist in the active site residues that lead to hypothesize different mechanisms towards β -lactam hydrolysis. In class D enzymes, this carbamoylated lysine is involved in both the acylation and the deacylation steps of the reaction [Schneider *et al.*, 2009]. The different substrate specificity of class D oxacillinases with respect to carbapenem-hydrolysing class D β -lactamases (CHDLs) has been shown to reside, at least in part, in the different extent and composition of the β 5- β 6 loop [Docquier *et al.*, 2009; De Luca *et al.*, 2011]. This aspect is relevant with respect to the carbapenemase function. Indeed, when the second structure of a CHDL was obtained (OXA-48), it was noticed that OXA-24, OXA-23 and OXA-48 differ from the other oxacillinases in the extent and orientation of the β 5- β 6 loop.

D) Metallo- β -lactamase structure:

MBLs belong to a larger superfamily of metalloproteins with diverse biological functions beyond β -lactam hydrolysis, designated as the metallo-hydrolase/oxidoreductase superfamily in the Structural Classifications of Proteins (SCOP) database [Murzin *et al.*, 1995; Baier and Tokuriki, 2014; Daiyasu *et al.*, 2001]. A common structural feature conserved in this superfamily is the overall $\alpha\beta/\beta\alpha$ protein fold, in which an internal mixed β -sheet sandwich is surrounded by solvent-exposed α -helices. Structural and sequence comparisons are consistent with the proposal that serine β -lactamases evolved from ancestral transpeptidases [Massova and Mobashery, 1998], but the evolutionary history of MBLs, which are not homologous to transpeptidases, is more ambiguous. Pseudo two-fold symmetry of the N- and C-terminal halves of MBLs suggests that the two halves may have arisen from an ancient gene duplication event [Massova and Mobashery, 1998; Carfi *et al.*, 1995]. In the MBLs, the zinc-containing active site is positioned within a shallow groove formed at the interface of the central β -sheets. Crystal structures of B1 β -lactamases reveal a mono or dinuclear metal ion cluster in which Zn1 is usually coordinated with tetrahedral geometry, and Zn2 with trigonal bipyramidal geometry when substrate or product ligands are absent [Concha *et al.*, 1996; Carfi *et al.*, 1995]. In the structures of monozinc B1 MBLs, the Zn1 site is occupied by the sole zinc ion. In structures of dizinc B1 MBLs with differing affinities for each site, Zn1 is typically refined with higher average occupancy than Zn2, consistent with biochemical studies assigning Zn2 as the more weakly binding site [Kim *et al.*, 2011; de Seny *et al.*, 2001; Paul-Soto *et al.*, 1999b; Rasia and Vila, 2002]. In other B1 MBLs, the dizinc site has been shown to bind both zinc ions with positive cooperativity, with binding of the first equivalent enhancing binding of the second (reviewed in [Aitha *et al.*, 2014]). “Second shell zinc ligands,” residues that directly interact with the primary zinc ligands, are more varied than the primary zinc ligands and may

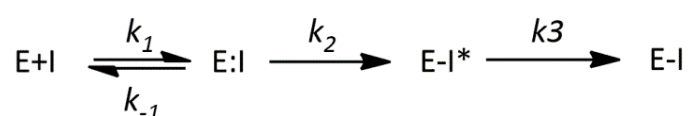
contribute to the differing properties found among the zinc sites of B1 MBLs [Meini et al., 2014; González et al., 2014; Crowder et al., 2006b; Tomatis et al., 2005]. In addition to the primary zinc ligands, several notable active-site features appear to be structurally conserved among the representative B1 MBLs IMP-1, VIM-1 and NDM-1, despite the relatively low overall amino acid sequence identities (VIM-1 vs IMP-1: 31%; NDM-1 vs IMP-1: 30%; VIM-1 vs NDM-1: 31%). Firstly, a positively-charged side chain (Lys224 in IMP-1 and NDM-1 or an Arg228 supplied from a different sequence position in VIM-2) is located approximately 6 Å from the Zn cluster and likely provides a counter ion for the conserved carboxylate found in all β -lactam drugs. Secondly, an Asn residue is structurally conserved in each of these three B1 MBLs, and this position (Asn233), found in active-site loop 10 (ASL10), is shown to be within H-bonding distance of the newly formed carboxylate in ring-opened β -lactam products. All of the residue numbering used here is standardized according to the standard BBL numbering [Garau *et al.*, 2004], which was also applied to NDM-1 [Cadag *et al.*, 2012]. Thirdly, in all three β -lactamases, a conserved extended -hairpin loop (active-site loop 3, ASL3) towers over one side of the active site, providing a broad hydrophobic surface for binding the core ring structures of the β -lactam substrates. In general, this loop is more disordered than the remainder of the protein [Concha et al., 2000; Garcia-Saez et al., 2008; King and Strynadka, 2011] and was demonstrated to become rigid and close down upon binding active-site ligands [Huntley et al., 2000; Scrofani et al., 1999; Huntley et al., 2003; Rydzik et al., 2014].

Palacios *et al.* have shown that loop L3 replacement in the scaffold of NDM-1 does not shape the substrate profile of this enzyme [Palacios *et al.*, 2018], but loop L3 does play an important role in the catalytic mechanism by tuning the rate of the rate-limiting step and controlling the stability of key reaction intermediates according to its conformation. A more closed loop would lead to an enhanced accumulation of the intermediate, while a more open active site would decrease the amount of intermediate. Thus, the substrate protonation step could be modulated by the solvent accessibility in different loop mutants, accounting for their different k_{cat} values.

Finally, in all three examples, a shallow groove in the protein surface extends away from either side of the dizinc cluster, allowing sufficient space to accommodate the varied substitutions found in the broad array of β -lactam substrates that contain structurally diverse substituents appended to either the four-membered lactam ring in one direction (away from Zn1), or extending from position 2 (in penams, clavams, penems and carbapenems) or position 3 (in cepheids) in the other direction (away from Zn2).

β -lactamase Inhibitors:

A successful strategy for combating β -lactamase-mediated resistance is the use of agents designed to bind at the active site, which are frequently β -lactams. This strategy can take two forms: (i) create substrates that reversibly and/or irreversibly bind the enzyme with high affinity but form unfavorable steric interactions as the acyl-enzyme (this is the case of the β -lactams already described as carbapenems or cephalosporins) or (ii) develop mechanism-based or irreversible “suicide inhibitors” [Bush, 1988]. Irreversible “suicide inhibitors” can permanently inactivate the β -lactamase through secondary chemical reactions in the enzyme active site. The following equation represents a general mechanism of irreversible inhibitors (I) leading to permanent enzyme inactivation (E-I*):



Irreversible inhibitors can be characterized by first-order rate constants for inhibition (k_{inact} , the rate of inactivation achieved with an “infinite” concentration of inactivator) and K_i values (the concentration of inactivator which yields an inactivation rate that is half the value of k_{inact}) [Copeland, 2005; Bush, 1986]. While K_i closely approximates the meaning of K_m for enzyme substrates, depending on the individual rate constants comprising the reaction, the K_i may or may not equal the equilibrium constant $K_i (= k_{-1}/k_1)$ determined under pre-steady-state conditions. The 50% inhibitory concentration (IC_{50}) measures the amount of inhibitor required to decrease enzyme activity to 50% of its uninhibited velocity. While an IC_{50} can reflect an inhibitor’s affinity or $k_{\text{cat}}/k_{\text{inact}}$ ratio, these parameters are not always congruent, e.g. an inhibitor can have a very poor “affinity” and acylate the enzyme slowly but still yield a low IC_{50} because of very low deacylation rates. The activity of an inhibitor can be evaluated by the turnover number (t_n) (also equivalent to the partition ratio [$k_{\text{cat}}/k_{\text{inact}}$]), defined as the number of inhibitor molecules that are hydrolysed per unit time before one enzyme molecule is irreversibly inactivated [Bush *et al.*, 1993]. An overview of inhibitors that are approved or in clinical development is presented in Figure 21.

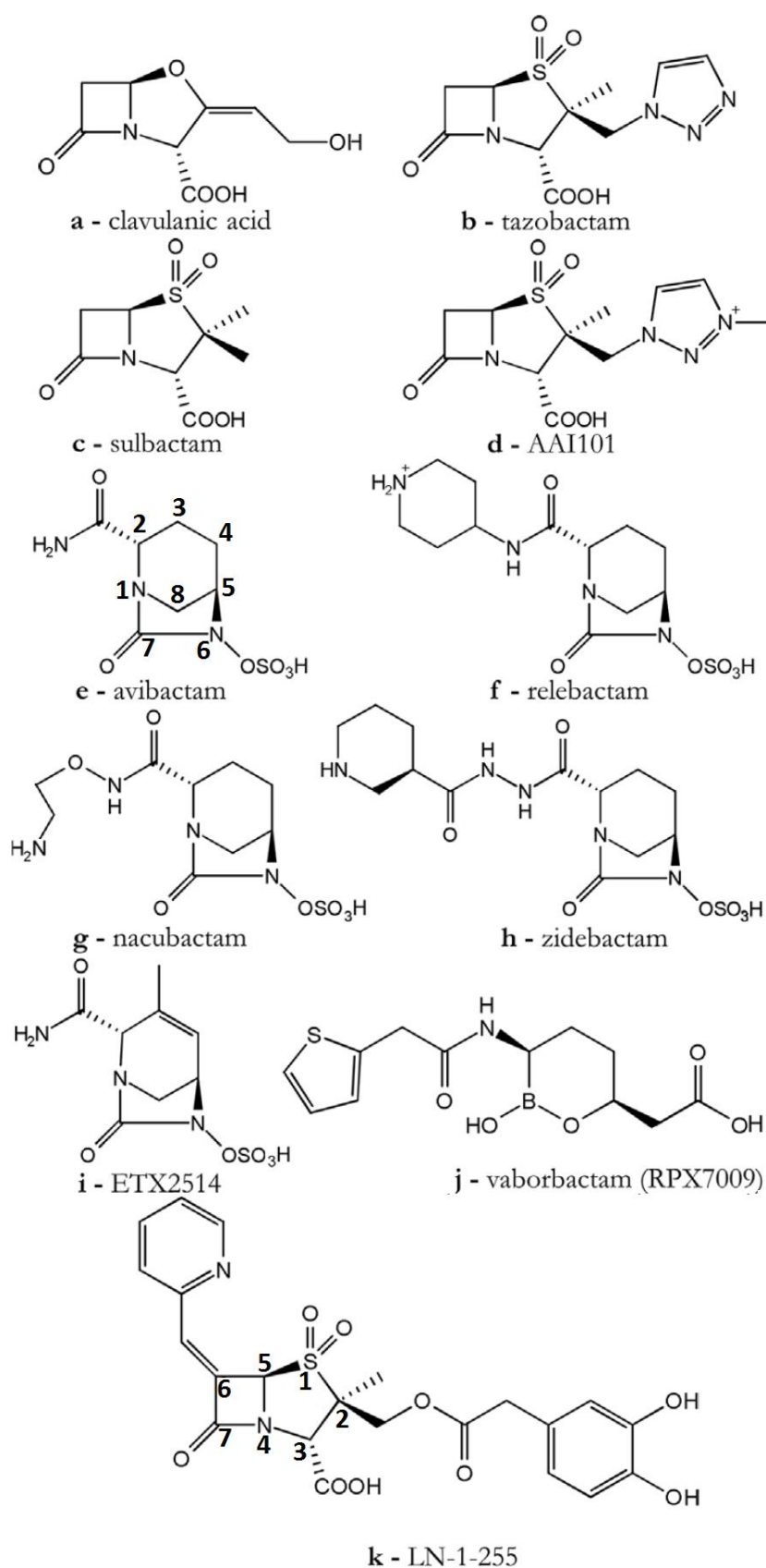


Figure 21. β -lactamase inhibitors. A) β -lactam compounds: a) clavulanic acid; b) tazobactam; c) sulbactam; d) AAI101; B) diazabicyclooctanone compounds: e) avibactam; f) relebactam; g) nacubactam; h) zidebactam; i) ETX2514; C) boronic acids: j) RPX7009; D) alkylidene penam sulfones: k) LN-1-255.

A) Oxapenem and penam sulphones:

Clavulanic acid (Figure 21a), the first β -lactamase inhibitor introduced into clinical medicine, was isolated from *Streptomyces clavuligerus* in the 1970s [Reading and Cole, 1977]. Clavulanate (the salt form of the acid in solution) showed little antimicrobial activity alone, but when combined with amoxicillin, clavulanate significantly lowered the amoxicillin MICs against *S. aureus*, *K. pneumoniae*, *P. mirabilis*, and *E. coli* [Brown, 1986]. Sulbactam (Figure 21c) and tazobactam (Figure 21b) are penicillinate sulfones that were later developed by the pharmaceutical industry as synthetic compounds in 1978 and 1980, respectively [English *et al.*, 1978; Fisher *et al.*, 1980].

Clavulanate, sulbactam, and tazobactam differ from β -lactam antibiotics as they possess a “leaving group” at position 1 of the five membered ring (sulbactam and tazobactam are sulfones, while clavulanate has an enol ether oxygen at this position), permitting a secondary ring opening and β -lactamase enzyme modification. Compared to clavulanate, the unmodified sulfone in sulbactam is a relatively poor “leaving group”, a property reflected in the high partition ratios for this inhibitor [Imtiaz *et al.*, 1993, 1994]. Tazobactam possesses a triazole group at the C-2 β -methyl position (see position numbering in Figure 7). This modification leads to tazobactam’s improved IC50s, partition ratios, and lowered MICs for representative class A and C β -lactamases [Buynak, 2006; Bonomo *et al.*, 1997; Bush *et al.*, 1993].

Generally, the inhibitors do not inactivate PBPs, but notable exceptions include (i) the intrinsic activities of sulbactam against *Bacteroides* spp., *Acinetobacter* spp., and *N. gonorrhoeae*; (ii) clavulanate action against *Haemophilus influenza* and *N. gonorrhoeae*; and (iii) tazobactam inhibition of PBPs in *Borrelia burgdorferi* [Urban *et al.*, 1991; Miller *et al.*, 1978; Bush, 1988; Higgins *et al.*, 2004; Levin, 2002; Neu and Fu, 1978; Urban *et al.*, 1993; Williams, 1997]. As these “antibacterial effects” are relatively weak, the inhibitors are always combined with β -lactam antibiotics for clinical use. Currently, there are five β -lactam/ β -lactamase inhibitor formulations available. Amoxicillin-clavulanate, ticarcillin-clavulanate, ampicillin-sulbactam, and piperacillin-tazobactam are available in the United States and Europe. Cefoperazone-sulbactam is used in several European countries, Japan, and India but is not available in the United States.

Following the success of sulbactam and tazobactam, medicinal chemists have focused much effort on the development of penicillin and cephalosporin sulfones with functionalities that may improve inhibitor efficiency. A close analogue of tazobactam, AAI101 (Figure 21d), entered clinical development in combination with cefepime [Crandon and Nicolau, 2015]. AAI101 is apparently a better inhibitor of KPC-type carbapenemases than tazobactam. Furthermore, the progressive addition of chemical substituents at positions 2 and 6 of penam and penem sulfones led to molecules with interesting properties, especially regarding the potential inhibition of class D β -lactamases, which are commonly less susceptible to available conventional inhibitors.

One of the first series of investigational sulfones, the C-7 alkylidene cepheems, had lead compounds that were potent inhibitors of class C β -lactamases [Buynak *et al.*, 2002]. Subsequent reports have explored the roles of C-2 and C-6 penam and C-3 cephem substituents (see position numbering in Figure 12). β -lactamase inhibitors with C-2 substitutions have shown efficacy against TEM- and SHV-type enzymes, including ESBLs [Richter *et al.*, 1996; Tzouvelekis *et al.*, 1997]. The 6-alkylidene-2'-substituted penicillin sulfone LN-1-255 (Figure 21k), attains nM KIs for the class A SHV-1 and ESBL SHV-2 and class D oxacillinase-, ESBL-, and even carbapenemase- type OXA enzymes [Buynak *et al.*, 1999; Pattanaik *et al.*, 2009]. The C-3 hydrophobic catechol moiety of this investigational compound appears

to improve both its entry into cells via siderophore iron channels and its affinity for β -lactamase active sites [Chakraborty *et al.*, 2007].

C-6-substituted penicillins are nM to low μ M inhibitors of serine β -lactamases, with the sulfone oxidation state at the penam thiazolidine sulfur showing improved affinities over the sulfide state [Bitha *et al.*, 1999; Buynak *et al.*, 2004; Sandanayaka *et al.*, 2001; Sandanayaka and Yang, 2000]. The stereochemistry and specific functionality of the C-6 substituents can affect the specificity of inhibition. For example, β isomer alkenyl derivatives, as opposed to α isomer derivatives, show improved IC₅₀s for class B β -lactamases, and derivatives with thiazole rings, particularly with fluorine, amino, or hydroxyl groups, show potency for class A β -lactamases [Sandanayaka *et al.*, 2001]. Further, compounds with a mercaptomethyl group at C-6, as opposed to hydroxymethyl, are better inhibitors of class B enzymes, while C-6-mercapto-penicillins are relatively less active inhibitors of classes A, B, and C [Buynak *et al.*, 2004]. When the hydrolytic water molecule approaches the acyl-enzyme from the “ α direction” (Figure 22), as with class A enzymes, α -hydroxyalkylpenicillins are effective inhibitors. Conversely, β -hydroxyalkylpenicillins inhibit the “ β approach” (Figure 22) in class C enzymes [Golemi *et al.*, 2000]. 6- β -hydroxymethyl penicillin sulfone has the best activity against both class A and C enzymes [Bitha *et al.*, 1999]. Presumably, the inhibitor worked like tazobactam or sulbactam in class A enzymes, but the specific crowding of the β -face of the ester prevents the approach of hydrolytic water in class C β -lactamases [A. Bulychev *et al.*, 1997]. The class D OXA-10 enzyme showed inhibition by both C-6 α - and β -hydroxyisopropylpenicillins [Maveyraud *et al.*, 2002]. While only the β -isomer could be crystallized with OXA-10 (PDB 1K54), this structure and computational models of the α -isomer suggest related, but different, mechanisms of inactivation based on the C-6 stereochemistry of the inhibitor. However, both mechanisms involved displacing the hydrolytic water necessary for deacylation.

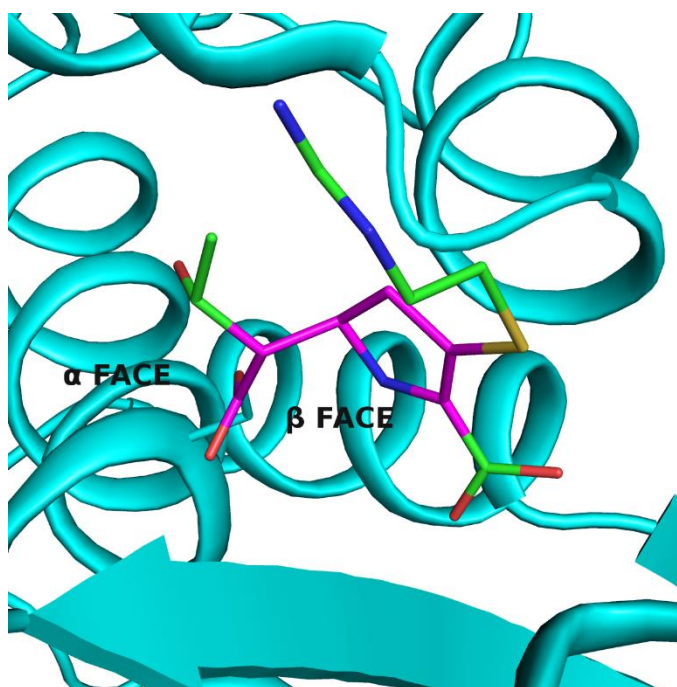


Figure 22. Hydrolytic water approach. Acyl-enzyme complex of OXA-48 and imipenem. Enzyme is depicted as cyan ribbon, and serine 70 and imipenem as sticks. Carbon atoms belonging to the fused β -lactam and penem rings are colored magenta for easier identification of the core structure. In serine- β -lactamases from classes A and D the hydrolytic water approaches the ester bond to hydrolyse the acyl-enzyme complex from the α face, whereas in class C enzymes, it comes from the β face.

Other modified β -lactam molecules have been investigated and contain a “siderophore” (iron-chelating) moiety [Page, 2013]. This approach allowed to obtain antibiotics characterized by efficient permeation in the bacterial cell by luring the iron uptake system. These molecular “Trojan horses” are usually characterized with a high antibacterial activity on Gram-negative organisms considered difficult due to the intrinsically low permeability of their outer membrane, especially *Pseudomonas* and *Acinetobacter* [Moynié *et al.*, 2017]. BAL19764, a dihydroxypyridone monosulbactam, was tested in combination with a bridged monobactam BAL29880 and clavulanate [Page *et al.*, 2011]. The clever rationale behind using modified monobactams was their stability to hydrolysis by metallo- β -lactamases and their inhibition of class C enzymes. Consequently, this triple combination (known as BAL30376) showed activity on carbapenem-resistant metallo- β -lactamase-producing organisms. Further modification of the molecule yielded its dihydroxypyridone monosulfactam analogue, BAL30072 [Higgins *et al.*, 2012]. This compound showed significant activity on a wide range of Gram-negative pathogens producing different kinds of β -lactamases [Hofer *et al.*, 2013; Mushtaq *et al.*, 2013].

Cefiderocol (S-649266) represents another siderophore mimic-containing β -lactam which also appears to be relatively stable to hydrolysis by clinically-relevant β -lactamases [Ito-Horiyama *et al.*, 2016; Ito *et al.*, 2016b; Kohira *et al.*, 2016]. From a structural standpoint, it is an oxyimino-cephalosporin in which the oxyimino group substituent (2-carboxypropyl) is that found in ceftazidime, while the C3 substituent is similar to that found in cefepime (1-methylpyrrolidinium) but includes a catechol (siderophore) moiety. It shows activity on *P. aeruginosa*, *A. baumannii* and carbapenem-resistant Enterobacteriaceae producing various types of carbapenemases (metallo- β -lactamases, KPC, NMC-A, OXA-48) [Dobias *et al.*, 2017; Falagas *et al.*, 2017; Ito *et al.*, 2016a].

In addition, the development of novel β -lactam/ β -lactamase combinations was also driven by the optimization of β -lactams with interesting properties. Ceftolozane-tazobactam is an example of combination between a new cephalosporin in which the addition of tazobactam is beneficial and grant protection from several common clinically-relevant enzymes. As a result, the combination shows a strong antibacterial activity on Gram-negative pathogens, and especially against drug-resistant *Pseudomonas aeruginosa* [Bush, 2012; Scott, 2016].

B) Diazabicyclo-octanones:

Based on the available knowledge regarding β -lactamase mechanism of action, scientists at Sanofi Aventis designed a small-sized molecular scaffold that would represent a structural mimicry of β -lactams, although it would be structurally different from a β -lactam, and thus would have the potential to escape the known mechanism of resistance of β -lactam-type β -lactamase inhibitors (BLIs) [Bonnefoy *et al.*, 2004a]. This molecular scaffold, the diazabicyclo-octanones (DBOs), allowed to identify avibactam (Figures 21e and 23) as a potent inhibitor of β -lactamases, with an inhibition spectrum that was considerably broadened when compared to that of clavulanate. Indeed, avibactam efficiently inhibits most class A β -lactamases, including the inhibitor-refractory KPC-type carbapenemases, both the chromosome and plasmid-encoded class C enzymes and several class D β -lactamases, including the OXA-48 carbapenemase [Ehmann *et al.*, 2013, 2012; Stachyra *et al.*, 2009; Bonnefoy *et al.*, 2004b; Livermore *et al.*, 2008; Mushtaq *et al.*, 2010]. The crystal structure of different β -lactamase-avibactam covalent adducts provided a structural basis for the broad spectrum of inhibition of the inhibitor [Lahiri *et al.*, 2013, 2015]. The avibactam molecule bound to the catalytic serine, in which the oxygen carbonyl is located in the oxyanion hole, creates a network of interactions that is very similar in both class A and class C enzymes and closely resembles that of acylated β -lactam

substrates. The sulfonate moiety interacts with residues of the conserved KTG motif, while the carboxamide side chain is stabilized by interactions with Asn/Gln residues essentially found in the SxN conserved motif and in the Ω -loop. The presence of two nitrogen atoms in the inhibitor allows these atoms to interact with catalytically relevant elements of β -lactamases. The mechanism of inhibition of avibactam is significantly different from that of clavulanate or penam sulfones, in that avibactam acts, with most β -lactamases, as a reversible covalent inhibitor (Figure 23) [Ehmann *et al.*, 2012]. The latter resides in the specific conformation adopted by the serine-bound avibactam in the β -lactamase active site, which shows significant differences with β -lactam substrates or inhibitors. In the avibactam molecule, the presence of a piperidine ring located “above”, rather than besides, the scissile C7(=O)–N6 bond is keeping the inhibitor in a relatively strained conformation. As a consequence, and despite the hydrolytic deacylation water molecule was present in most X-ray structures of the complexes, the N6 atom of avibactam is a better nucleophile than the hydrolytic water, resulting in the (slow) formation of the original molecule and the release of the free enzyme [Lahiri *et al.*, 2013, 2015; Ehmann *et al.*, 2013].

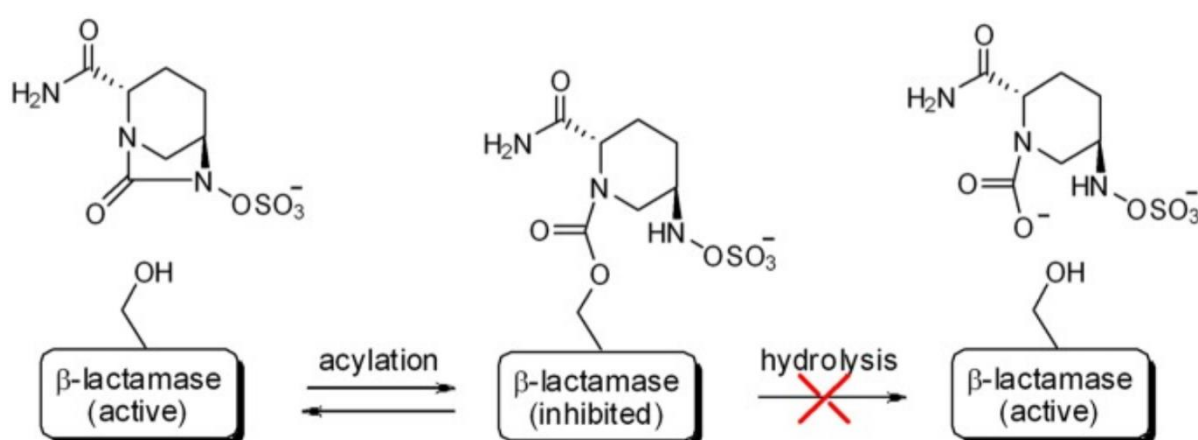


Figure 23. Inhibitory mechanism of Avibactam.

Relebactam (MK-7655, Merck, Figure 21f), showing a piperidinium substituent on the carboxamide side chain of the DABCO scaffold, is currently being developed in combination with imipenem-cilastatin [Blizzard *et al.*, 2014]. The inhibitor shows properties very similar to that of avibactam except a lower activity on OXA-48-like carbapenemases. Being developed in combination with a carbapenem, rather than with an oxyimino-cephalosporin, it however shows a slightly different profile of antibacterial activity, especially with OXA-type-producing Enterobacteriaceae [Bush and Bradford, 2016].

Nacubactam (RG6080, FPI1459, OP0595, Figure 21g), another DABCO showing a substituent on the carboxamide side chain (in this case, a 2-aminoethoxy group), interestingly shows a potent inhibition of both β -lactamases (IC₅₀ values as low as 10 nM) and PBP2 (IC₅₀, 370 nM) [Morinaka *et al.*, 2015; Livermore *et al.*, 2016]. Nacubactam shows a remarkable synergy with various β -lactams, translating in a so-called “enhancer” effect.

Zidebactam (Figure 21h), currently developed in combination with cefepime, is another example of substituted DABCO with a similar enhancer effect, again thanks to a significant inhibition of PBP2 [Moya *et al.*, 2017].

Another strategy, besides the diversitification of the carboxamide substituent, regarded the modification of the piperidine ring of the inhibitor and led to the discovery of the more recent DABCO

inhibitor, ETX2514 (Figure 21i). This compound also shows PBP2 inhibition while keeping a potent inhibitory activity towards β -lactamases, with an intrinsic antibacterial activity against Enterobacteriaceae (MIC₉₀, 1–8 μ g/ml, on *E. coli* and *K. pneumoniae* isolates) [Durand-Réville *et al.*, 2017; Shapiro *et al.*, 2017]. ETX2514 was rationally designed to improve its reactivity towards β -lactamases and its diffusion through the outer membrane of Gram-negatives, without affecting its stability. When compared to relebactam or nacubactam, ETX2514 shows a more rapid and effective inhibition of all tested serine- β -lactamases, with a large improvement on class D β -lactamases, including the *Acinetobacter* OXA-24 carbapenemase. Exploiting the moderate intrinsic activity of sulbactam on *Acinetobacter* spp., the combination sulbactam/ETX2514 showed a good in vitro activity on multidrug-resistant *Acinetobacter baumannii* (MIC₉₀, 4 μ g/ml; 191 clinical isolates with resistance to carbapenems, ceftazidime, aztreonam and sulbactam).

C) Boronic acid transition state analogs:

In the 1970s, boronic acid compounds were described as forming reversible, dative covalent bonds with serine proteases and inhibiting these enzymes by assuming tetrahedral reaction intermediates [Bone *et al.*, 1987; Lindquist and Terry, 1974]. These compounds, which lack the β -lactam motif, form adducts that resemble the geometry of the tetrahedral transition state of the β -lactamase hydrolytic reaction, either MBLs or SBL [Beesley *et al.*, 1983; Chen *et al.*, 1993; Crompton *et al.*, 1988]. Working via a novel mechanism compared to that of the clinically available β -lactamase inhibitors, boron forms a reversible dative bond with the β -lactamase, is not hydrolyzed by the enzyme, and serves as a competitive inhibitor (Figures 21 and 24). Glycylboronates are based on β -lactam substrate homology and contain side chains of penicillins and cephalosporins. Studies have shown analogues of ampicillin, cephalothin, and cefoperazone to be inhibitors of clinically relevant class A and C β -lactamases in the nM range [Winkler *et al.*, 2013; Ke *et al.*, 2011; Drawz *et al.*, 2010, 2011b]. These compounds are still preclinical. More recently, new modifications to the boronate analogue of β -lactam were added by replacing the carboxamide group, conserved in all penicillin and cephalosporins, with a sulfonamide [Eidam *et al.*, 2010; Tondi *et al.*, 2010]. These novel derivatives result more potent than their carboxamide analogues. Combined with ceftazidime, the lead compounds lowered MICs up to 64-fold.

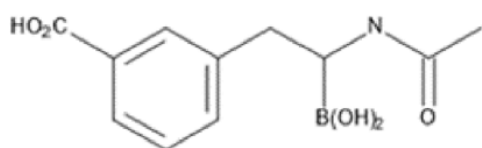
Human serine hydrolases have, in general, sterically constrained active sites, better suited to host linear compounds, while β -lactamases have wider active sites able to bind cyclic molecules mimicking tetrahedral transition states. Hecker and co-workers considered the possibility to achieve higher selectivity towards β -lactamases, without losing affinity by screening cyclic boronate compounds [Hecker *et al.*, 2015].

Alone, RPX7009 (vaborbactam, Figure 21j) did not exhibit antibacterial activity, but the combination showed strong potentiation of biapenem against class A carbapenemase-producing Enterobacteriaceae. The properties of RPX7009 led to successful completion of phase 1 trials alone (vaborbactam) [Griffith *et al.*, 2016]) and in combination with either biapenem (RPX2003; biapenem; ClinicalTrials.gov Identifier: NCT01772836) [[Livermore D *et al.*, 2012; Castanheira *et al.*, 2012; Sabet *et al.*, 2012; Hecker *et al.*, 2012] or meropenem (Carbavance [Vabomere]; Clinical-Trials.gov Identifiers: NCT02020434, NCT02073812 and NCT01897779). These allowed the latter combination to enter a clinical phase 3 trial in combination with meropenem and to receive FDA approval in 2017.

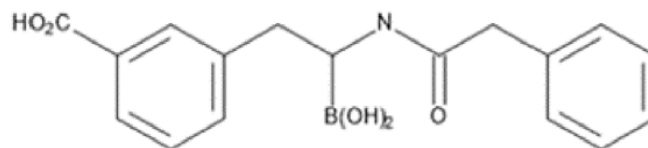
Noteworthy, the ability of boric acid, borate anions, boronic acids to also act as inhibitors of mononuclear and dinuclear metallo-enzyme inhibition has been exploited either for mechanistic studies and for development of new drugs.

Scientists at Rempex Pharmaceuticals (then the Medicines Company) were able to introduce further chemical modifications in the inhibitor molecule to obtain a substantial improvement of their inhibitory activity on metallo- β -lactamases [Hecker *et al.*, 2015]. A first optimization of the inhibitor was achieved with the introduction of an aminothiadiazoole side chain in RPX7262 (Figure 24g), which kept nanomolar potency against serine carbapenemase and a promising inhibition of the NDM-1 metallo- β -lactamase (K_i , 7.4 μ M). However, the most significant increase in the potency of inhibition towards metallo- β -lactamases was achieved with a more drastic modification of the molecule, in which the amide group was substituted by a sulphur atom, followed by a similar thiadiazoole moiety. RPX7282 showed pan-spectrum inhibition of β -lactamases, with a potent and similar activity against both serine- and metallo-carbapenemases (K_i , 0.01–0.03 μ M) [Hecker *et al.*, 2015]. RPX7282 (Figure 21i) showed a good synergistic activity with carbapenems, and were notably able to restore carbapenem susceptibility with VIM-2- or NDM-1-producing strains at inhibitor concentrations as low as 0.3 μ g/ml [Lomovskaya *et al.*, 2015].

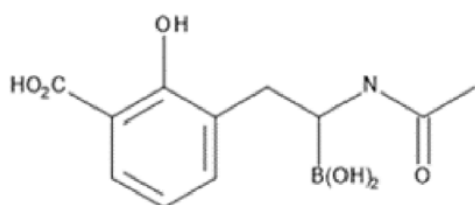
Schofield *et al.* have followed this path by testing a variety of boronic acids, some of which were already known SBL or PBP inhibitors, in search of inhibitors of MBL enzymes like NDM-1 [Brem *et al.*, 2016b]. They characterized cyclic boronates that interestingly displayed inhibitory activity against SBL, MBL and PBP enzymes. Among them, they identified a compound already described in the patent literature (Figure 24i) [Burns *et al.*, 2010] which has the highest inhibition towards members of all three enzyme classes and has the ability to restore meropenem activity against Gram-negative bacterial strains carrying both SBL and MBL genes [Brem *et al.*, 2016b].



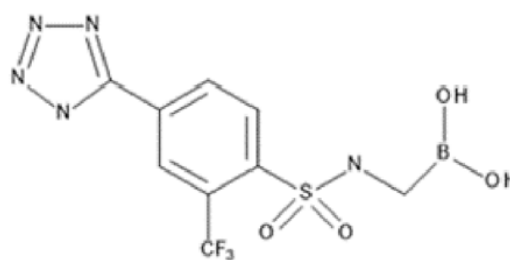
a) PDB code 1ERM



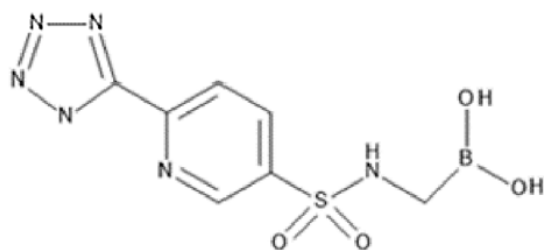
b) PDB code 1ERO



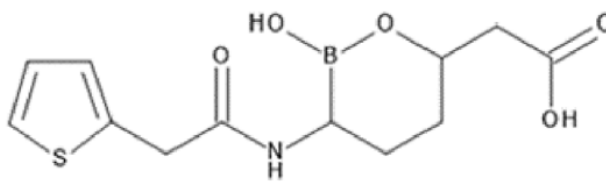
c) PDB code 1ERQ



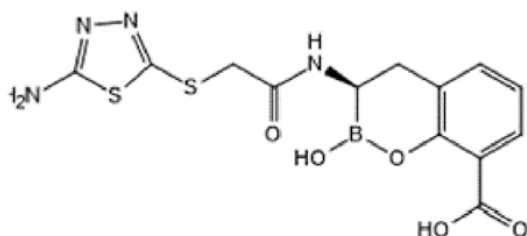
d) PDB code 4E3N



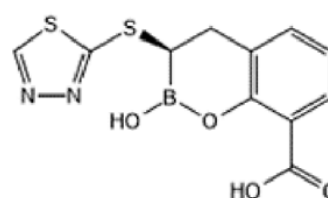
e) PDB code 4E3K



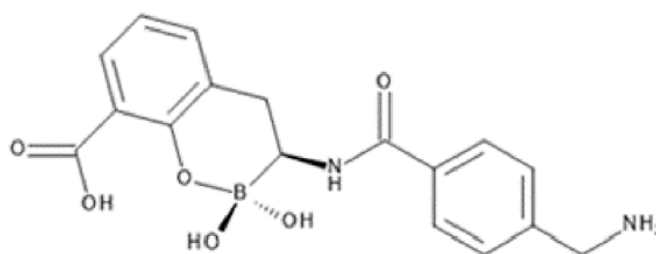
f) RPX-7009



g) RPX-7262



h) RPX-7282



i) Patent WO 2010/130708 A1

Figure 24. Boronic acid-based β -lactamase inhibitors. a-e) unnamed components present in β -lactamase/inhibitor complex structures in the PDB, f) RPX7009, g) RPX-7262, h) RPX-7282, i) compound from patent literature (Patent WO 2010/130708 A1)

D) Phosphonates:

Phosphonate monoester derivatives can acylate the active-site serine of class A, C, and D β -lactamases, leading to effective inhibition [Adediran *et al.*, 2005; Li and Pratt, 1998; Majumdar and Pratt, 2009; Pratt, 1989; Majumdar *et al.*, 2005]. These compounds exhibit branched kinetic pathways, in some cases reflecting inhibition by the products that are formed. Acyl phosphonates inhibit class C enzymes, while cyclic acyl phosphonates inhibit both class A and C β -lactamases. For diacyl phosphonates, hydrophobic substituents decrease the inhibitor's K_i value, and the acylation rates of these compounds follow the order class D > class C > class A [Majumdar *et al.*, 2005; Majumdar and Pratt, 2009]. This relative inhibitor efficiency reflects the general hydrophobicity of the enzyme active sites and that rational design of diacyl phosphate side chains could lead to nM inhibition of all serine β -lactamase classes. The clinical potential of phosphonates has been limited by their poor stability in aqueous solution and susceptibility to phosphodiesterases.

E) Metallo- β -lactamase inhibitors (MBLs):

MBLs are characterized by a broad substrate specificity, being able to hydrolyze all β -lactam antibiotics, most importantly carbapenems, with the exception of monobactams (e.g. aztreonam) [Walsh *et al.*, 2005; Bebrone, 2007]. MBLs are insensitive to most of the clinically available β -lactamase inhibitors and hence represent one of the most worrying bacterial resistance factors. The difficulty in finding a 'universal' inhibitor of MBLs arises from the structural and mechanistic differences spread among the 3 subclasses of MBLs recognized up to now and that have been classified on the basis of structural similarity [Garau *et al.*, 2004; Bebrone, 2007]. All MBLs share a common $\alpha\beta\beta\alpha$ fold. This protein fold is widely present in Nature, from bacteria to humans, and it is characteristic of a superfamily of dinuclear metallo-enzymes catalysing a variety of chemical reactions and acting on different substrates (for specialized reviews see [Bebrone, 2007]). MBLs inhibitors can be grouped depending on their chemical determinants able to interfere with the MBLs active site (Figure 25).

a. Zn binding inhibitors:

L-captopril (Figure 25b), a potent angiotensin converting enzyme (ACE) inhibitor, is a widely used drug for the treatment of hypertension and contains thiol and carboxylate groups able to establish coordination bonds with metal ions. These chemical determinants are responsible for the quite strong inhibitory effect of L- and D-captopril (Figure 25c) on MBLs of all subclasses (BclI, NDM-1, CphA, L1, FEZ-1) that has been established since a quite long time [García-Saez *et al.*, 2003; García-Sáez *et al.*, 2003; King *et al.*, 2012; Brem *et al.*, 2016a; Nauton *et al.*, 2008]. Captopril stereoisomers always bind the enzymes with the sulfur atom that replaces the bridging water/hydroxide of the dizinc cluster [Brem *et al.*, 2016a]. Klingler and co-workers proposed using already approved drugs that can be possibly repositioned for antibacterial therapy and succeeded in finding, besides captopril, three molecules having IC₅₀ in the low μ M range for NDM-1, IMP-7 and VIM-1: thiorphan (Figure 25d), dimercaprol (Figure 25e) and tiopronin (Figure 25f) [Klingler *et al.*, 2015].

Arjomandi *et al.* proposed [Arjomandi *et al.*, 2016] new thiol derivatives of tyrosine (Figure 25g) that constitute the development of the original compound proposed by Lienard *et al.* [Liénard *et al.*, 2008] as IMP-1 inhibitors. Other mercaptoacetic acid thioester amino acid derivatives (Figure 25h) active against NDM-1, ImiS-1 and L1 have been reported [Liu *et al.*, 2015]. Docking studies suggest that the

binding of these compounds to L1 Zn²⁺ ions occurs by the mercaptoacetic carboxylate group assisted by hydrogen bond to Ser221. A paper by the Spencer and Schofield groups [Rydzik *et al.*, 2014] describes a molecule with a thiazolidine core (Figure 25i) acting as a precursor of (2Z)-2- sulfanyl-3-(2,3,6-trichlorophenyl)2-propenoic acid (Figure 25j), a thioenolate inhibitor generated by the hydrolysis of the parent molecule. The crystal structure (PDB: 4PVO) shows the inhibitor chelating the Zn²⁺ ion of VIM-2 by its thiolate sulfur and by the carboxylate groups formed upon hydrolysis of the thiazolidine ring. The sulfur atom of the inhibitor is further bound to Zn1. The same occurs in the complex with BclI MBL (PDB: 4TYT). Soon after this paper, a novel scaffold based on thiazolidines appeared in the literature. This consists of bithiazolidines (BTZ) that present analogies with the β -lactams. Two papers report the crystal structure of the VIM-2 [Mojica *et al.*, 2015] and NDM-1 [González *et al.*, 2015] complexes (PDB: 4UA4 and 4U4L, respectively) with the L-isomer of a bithiazoline inhibitor (Figure 25k). Both structures show the inhibitor bound to the dizinc center by the only thiol/thiolate group bridging the two Zn²⁺ ions. The promising aspect of the thiadiazolidine scaffold is the ability to inhibit metallo- β -lactamases of all subclasses. However, from the structural analysis it appears that the cross-class inhibition is essentially due to the zinc binding capability of all compounds through the thiolate sulfur (only D-CS319 chelates Zn²⁺ by sulfur and carboxylate groups). Consequently, increasing the affinity by introducing groups able to establish H-bonds and/or hydrophobic interactions with the surrounding residues, if successful, will probably increase binding at the expense of inhibition spectrum due to the scarcity of common amino acid motifs in MBL active sites. Other compounds containing carboxylate groups able to inhibit MBLs with a sufficient selectivity were indeed previously described. The maleic acid derivative ME1071 was shown to potentiate the activity of carbapenems and cephalosporins on MBL-producing clinical isolates. Further carboxylate-bearing inhibitors were synthesized by Hiraiwa and co-workers [Hiraiwa *et al.*, 2014], like 3-(4-hydroxypiperidine-1-yl)phthalic acid (Figure 25o). New derivatives of this scaffold were obtained, displaying K_i below 1 μ M and having synergistic effect with the carbapenem biapenem on IMP-1-producing *P. aeruginosa* clinical isolates. Finally, 1,2,4-triazole-3-thione derivatives have been recently investigated for their ability to inhibit clinically-relevant metallo- β -lactamases [Sevaille *et al.*, 2017]. The mode of binding of two initial compounds was investigated in the L1 enzyme and showed unexpected differences between the two compounds [Nauton *et al.*, 2008; Sevaille *et al.*, 2017]. This subsequently allowed a new chemical series with some compounds showing a broad-spectrum inhibition.

b. Zn chelating inhibitors:

Strong chelating agents, as for example EDTA, can sequester and extract zinc from MBLs. Although effective, such compounds strongly suffer from being non-specific, and thus potentially associated with cytotoxicity issues. However, the potential clinical application of Ca-EDTA was demonstrated in a murine sepsis model of infection [Yoshizumi *et al.*, 2013]. The phytotoxin aspergillomarasmine A (AMA, Figure 25p,q), a product of pathogenic fungi, was reported to efficiently inactivate clinically-relevant metallo- β -lactamases like VIM-2 and NDM-1 [King *et al.*, 2014]. Aspergillomarasmine A is a derivative of L-aspartic acid and contains four carboxylates groups, with similar Zn-chelating properties as EDTA. Falconer and co-workers explored a series of spiro-indoline-thiadiazole compounds for their potential to inhibit MBLs [Falconer *et al.*, 2015]. SIT-5Z emerged as a moderately potent inhibitor of NDM-1, although its activity on other MBLs (VIM-2 and IMP-7) was lower in combination with meropenem. This inhibitor significantly reduced the bacterial load in both the liver and spleen of mice infected by an NDM-1-producing *K. pneumoniae* strain (peritoneal infection).

c. MBL covalent inhibitors:

An unusual type of inhibitor is the selenium-bearing compound ebselen (2-phenyl-1,2-benzoselenazol-3-one, Figure 25t) that has been shown to efficiently inactivate NDM-1 and restore meropenem activity on NDM-1 expressing *E. coli* cells [Chiou *et al.*, 2015]. It appears that the activity of this molecule is due to the opening of the selenazol ring and formation of a covalent Se-S bond with the zinc binding Cys221 residue. Along this line of research 4-oxo-4H-1-benzopyran-3-carboxaldehyde (3-formylchromone, Figure 25u) has also been shown to be a covalent inhibitor NDM-1 by binding to the active site Lys224 [Christopeit *et al.*, 2016]. Some β -lactams might undergo additional chemistry after being hydrolyzed by MBLs, progressing towards a covalent binding to the enzyme, and thus its inactivation. Moxalactam [Zervosen *et al.*, 2001] is able to combine with the Cys221 active site residue and cefaclor, an early cephalosporin, can cause chemical modification of the active lysine Lys224, rather than with the cysteine residue.

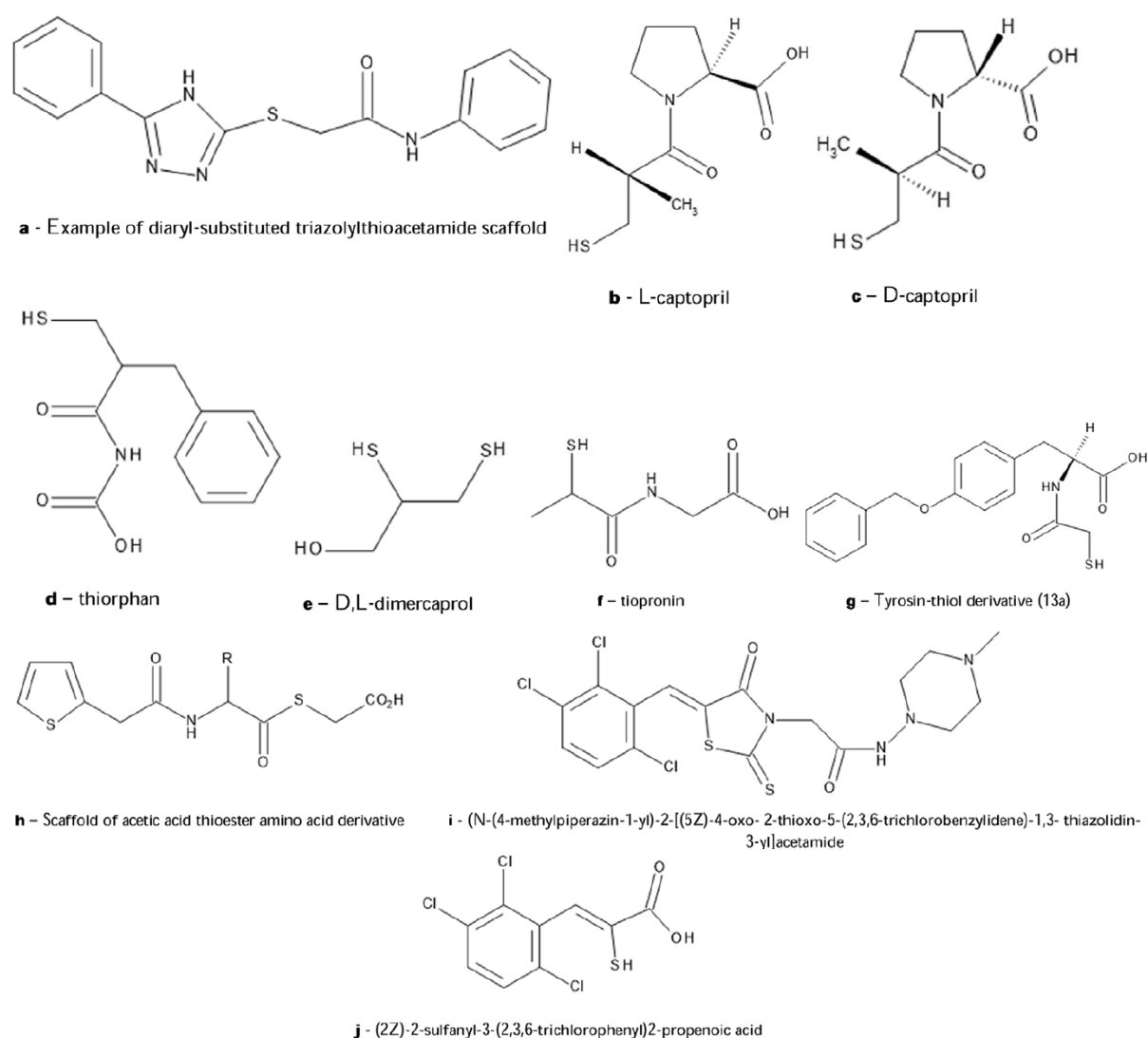


Figure 25. MBL inhibitors. a-o: Zinc binding inhibitors; p-s: Zinc chelating inhibitors; t-u: Covalent inhibitors

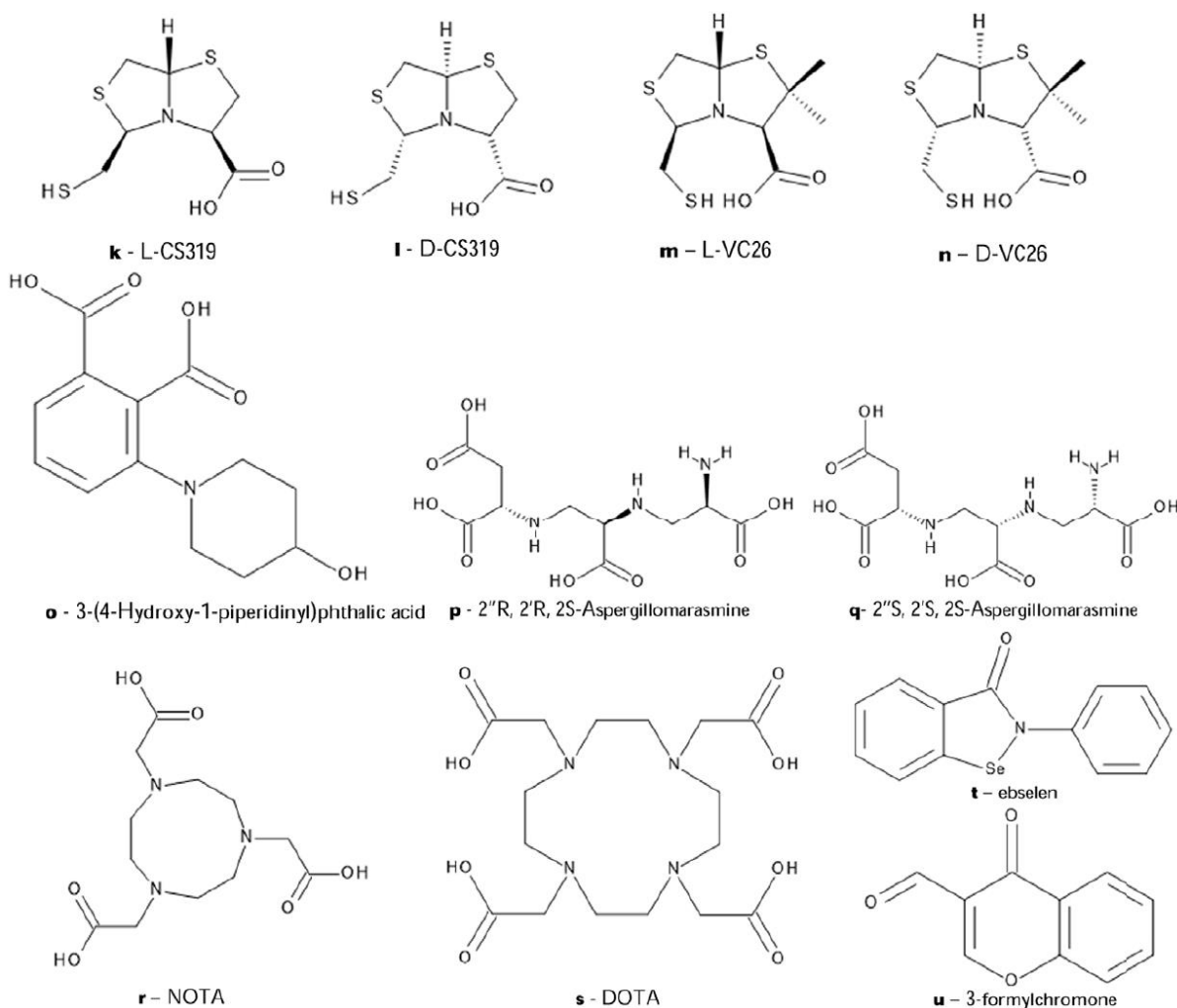


Figure 25. MBL inhibitors (continued). a-o: Zinc binding inhibitors; p-s: Zinc chelating inhibitors; t-u: Covalent inhibitors

X-ray crystallography and in-silico methodologies:

A) X-ray crystallography:

X-ray crystallography relies on the scattering of X-rays by the electrons in the molecules constituting the investigated sample. Because the highly similar structural motifs forming the individual unit cells are repeated throughout the entire volume of a crystal in a periodic fashion, it can be treated as a 3D diffraction grating. As a result, the scattering of X-radiation is enhanced enormously in selected directions and extinguished completely in others. This is governed only by the geometry (size and shape) of the crystal unit cell and the wavelength of the X-rays, which should be in the same range as the interatomic distances (chemical bonds) in molecules. However, the effectiveness of interference of the diffracted rays in each direction, and therefore the intensity of each diffracted ray, depends on the constellation of all atoms within the unit cell. In other words, the crystal structure is encoded in the diffracted X-rays – the shape and symmetry of the cell define the directions of the diffracted beams, and the locations of all atoms in the cell define their intensities. The larger the unit cell, the more diffracted beams (called ‘reflections’) can be observed. Moreover, the position of each atom in the crystal structure influences the intensities of all the reflections and, conversely, the intensity of each individual reflection depends on the positions of all atoms in the unit cell [Wlodawer *et al.*, 2008].

The primary result of an X-ray diffraction experiment is a map of electron density within the crystal. This electron distribution is usually interpreted in (chemical) terms of individual atoms and molecules, but it is important to realize that the molecular model consisting of individual atoms is already an interpretation of the primary result of the diffraction experiment. Finally, the atomic model is 'refined' by varying all model parameters to achieve the best agreement between the observed reflection amplitudes (F_{obs}) and those calculated from the model (F_{calc}).

The refinement process usually involves alternating rounds of automated optimization (e.g. according to least-squares or maximum-likelihood algorithms) and manual corrections that improve agreement with the electron-density maps. These corrections are necessary because the automatically refined parameters may get stuck in a (mathematical) local minimum, instead of leading to the global, optimum solution. The model parameters that are optimized by a refinement program include, for each atom, its x, y and z coordinates, and a parameter reflecting its 'mobility' or smearing in space, known as the B-factor (or displacement parameter, sometimes referred to as 'temperature factor'). A protein molecule of 20 kDa would take about 6000 parameters to refine, and, frequently, the number of observations (especially at low resolution) is not quite sufficient. For this reason, refinement is carried out under the control of stereochemical restraints which guide its progress by incorporating prior knowledge or chemical common sense [Hendrickson, 1985; Kleywegt and Jones, 1995]. The most popular libraries of stereochemical restraints (their standard or target values) have been compiled based on small-molecule structures [Engh *et al.*, 1991; Engh and Huber, 2006; Allen, 2002] but there is growing evidence from high-quality protein models that the nuances of macromolecular structures should also be taken into account [Jaskolski *et al.*, 2007]. Another way of model refinement, introduced more recently into macromolecular crystallography, involves dividing the whole structure into rigid fragments and expressing their vibrations in terms of the so-called TLS parameters which describe the translational, librational and screw movements of each fragment [Painter and Merritt, 2006]. Selection of rigid groups should be reasonable, corresponding to individual (sub)domains, for example. Although many of the steps in crystal structure analysis have been automated in recent years, the interpretation of some fine features in electron-density maps still requires a significant degree of human skill and experience [Brändén and Alwyn Jones, 1990]. A degree of subjectivity is thus inevitable in this process and different people working with the same data may occasionally produce slightly different results.

In the case of β -lactamases, X-ray crystallography has proven to be a powerful technique and has been extensively used to characterize novel β -lactamase enzymes [Lassaux *et al.*, 2011], seek structural basis for hydrolytic profile changes observed for novel naturally occurring variants [Crichlow *et al.*, 1999], structurally analyse rationally designed β -lactamase mutants [De Luca *et al.*, 2011], analyse binding modes of substrates or inhibitors [King *et al.*, 2012; Krishnan *et al.*, 2015], investigate the catalytic mechanism [Beadle *et al.*, 2002], or drive fragment-based drug design [Lund *et al.*, 2016] (Figure 26).

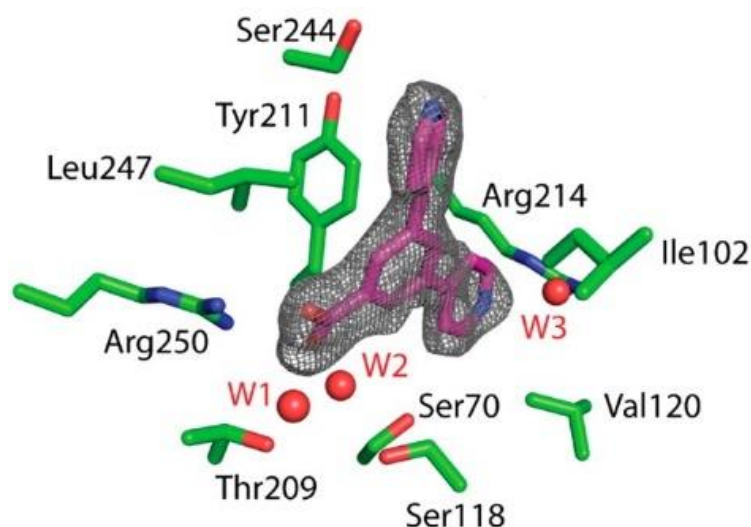


Figure 26. Crystal structure of an inhibitor (magenta) in complex with OXA-48 (green). The $F_o - F_c$ map is contoured at $+2.5\sigma$. Image from [Lund *et al.*, 2016].

B) Molecular Docking:

a. Docking of small molecules to proteins

Molecular docking studies are used to understand the interaction between a ligand and a target protein at the atomic level [Malathi and Ramaiah, 2018a]. Molecular docking is concerned with the determination of the optimal position(s) and orientation(s) of a small molecule in a protein target binding site [Śledź and Caflisch, 2018; Sotriffer, 2018]. It has been reported that while the success of the approach is target-dependent and software suite-dependent, it poorly correlates with the binding affinity but rather depends on the quality of interactions that the ligand makes to the protein [Verdonk *et al.*, 2011].

Importantly, the probability of successful prediction of the binding mode decreases substantially as the intrinsic flexibility of the ligand grows [Cecchini *et al.*, 2004], and depends on high-quality interactions made with the receptor [Verdonk *et al.*, 2011]. Thus, predictive ability has been validated for rigid fragments [Huang and Caflisch, 2009; Spiliotopoulos and Caflisch, 2016], while docking of peptides with more than a dozen rotatable bonds is considered speculative [Śledź and Caflisch, 2018]. A high degree of convergence toward the same pose in multiple docking runs of the same ligand (with different initial random populations of the genetic algorithm) was reported as necessary condition for successful prediction of the binding mode [Cecchini *et al.*, 2004].

Two operations are involved in the docking process, a search of conformational space available to a ligand followed by a scoring function representing binding affinity [Lohning *et al.*, 2017]. The molecular docking uses a stochastic, or random, searching algorithm and thus, can be limited by the time allocated to the search. There is an obvious trade-off between accuracy and time since the longer the process is allowed to proceed, the more likely it will be to find the global minimum conformation. This assumes that the lowest energy conformation is indeed the biologically relevant orientation. However, this is not always the case, as in transition state models.

Three key limitations of molecular docking are the inability to accurately model solvent, entropy and target flexibility. The treatment of water molecules remains a challenge, as it is difficult to predict

which solvent molecules are obligate in the binding site, and which can be displaced by incoming ligand. The water stability in the binding site can be determined based on analysis of multiple crystal structures, or more thoroughly by running MD simulations with explicit solvent [Huang *et al.*, 2014].

Docking methods play a central role in structure-based design, whether in virtual screening, for *de novo* design or at later stages of hit or lead optimization [Sotriffer, 2018].

b. Virtual screening by high-throughput docking

Among the most common molecular modeling techniques, molecular docking provides a convenient way to leverage structure for ligand discovery. Compared to laboratory-based, serendipitous, high throughput screening (HTS), molecular docking can access far more chemistry more quickly and with far less cost [Roy *et al.*, 2010].

‘Structure-based’ screens typically use molecular docking programs to fit small organic molecules into protein structures, evaluating them for structural and chemical complementarity [Kolb *et al.*, 2009]. The main challenge is not to identify the few nanomolar binders in the small-molecule library (if any at all), but rather to reduce the number of false negatives in the subset of compounds that are selected for validation by *in vitro* assays [Śledź and Caflisch, 2018]. Several million molecules may be docked into the structure of the target protein, and those that fit best, according to the docking scoring function, will be tested experimentally. Although the scoring functions retain substantial inaccuracies, the focus on commercially available molecules has made failure cheap — since one can always just purchase and test the next set of compounds — and so pragmatic [Kolb *et al.*, 2009].

c. Searching algorithms and scoring functions

The sampling algorithms are available in currently used docking software. Searching algorithms available in currently used docking software are: (i) matching algorithms; (ii) incremental construction method; (iii) Multiple Copy Simultaneous Search (MCSS); (iv) LUDI; (v) Monte Carlo; (vi) genetic algorithms [Malathi and Ramaiah, 2018b].

The main goal of the scoring function is to estimate the binding affinity of a compound with the protein and rank the complex against other candidates. These scoring functions are classified as ‘empirical’, ‘force field’ and ‘knowledge-based’. The empirical scoring functions involve the conversion of protein-ligand binding energy into many energy components. All of these components are multiplied and summed for the final score. Force field functional scorings estimate the binding affinity by the calculation of electrostatic and van der Waals interactions. Leonard-Jones potential energy was used for the van der Waals interactions and Columbic potential energy formulation was used for the electrostatic interactions. Knowledge-based scoring functions are emerging as an alternate method of grading protein-ligand binding affinities along with 3D structures.

d. Binding site flexibility

Protein flexibility constitutes another challenge of computer-aided drug discovery. Since the advent of structural biology methods and their application in structure-based drug discovery [Śledź and Caflisch, 2018], it has become apparent that many protein binding sites cannot be represented as a single snapshot, as significant structural rearrangements take place to accommodate diverse ligands. Information on binding site flexibility, available from experimental and/or simulation studies, can be taken into account to prepare one or more conformations of the target protein for (high-throughput) docking [Xu *et al.*, 2016].

The docking process is remarkably fast if target atoms are kept rigid, though the more patient user can expect higher quality results when the target incorporates flexibility in protein side-chains. Depending on the computer system, target biomolecule and number of ligands used in these calculations, a single flexible docking can be performed in around one minute [Steinbrecher and Labahn, 2010; Halperin *et al.*, 2002].

e. Computer programs for flexible ligand docking

There is a plethora of software suites developed for the automatic docking of flexible small molecules into (mainly rigid) protein structures. The most popular docking tools share similar sampling procedures (genetic algorithms-based optimization in the conformational space of the rotatable bonds or grid-based searches) and some of them use force field-based evaluation of the binding energy. Few docking programs have gained broad recognition and are used by a large community [Chen, 2015]. These include Dock [Allen *et al.*, 2015], GOLD [Jones *et al.*, 1997], and AutoDock [Morris *et al.*, 2009]. More recently, rDock has emerged as an efficient docking tool distributed as open source code [Ruiz-Carmona *et al.*, 2014].

f. Covalent docking

Drug discovery projects have traditionally focused on non-covalent ligands, i.e. compounds that interact with their target protein only through non-covalent interactions. This may appear surprising given that some of the most prominent drugs in history (such as aspirin or β -lactam antibiotics) are actually covalent binders [Sotriffer, 2018]. On the other hand, electrophilic compounds able to undergo covalent reactions with protein functional groups have generally been associated with too high risks for unspecific side effects and toxicity issues.

It is nowadays widely appreciated that covalent ligands can indeed be highly specific, and not necessarily associated with toxic side effects and offer, thus, new routes to compounds with high potency and prolonged duration of action.

The aim of docking is, however, binding-mode prediction, and the first goal for covalent docking approaches is to establish an appropriate handling of covalent bond formation in the context of the docking process. Most programs with functionalities for covalent docking are restricted to sampling ligand conformations under the constraint of a predefined bond between ligand and protein.

The oldest and still most common approach for covalent docking is the (pre-)formation of the covalent bond as a direct connection between the protein and the ligand prior to the actual docking process. This means that a link atom or a geometric constraint is defined to keep the electrophilic ligand atom in proximity or actually connected to the protein nucleophile. The docking process is thereby reduced to sampling energetically favourable conformations under the constraint of this preformed bond or a superposition ensuring a corresponding structural arrangement. Energy contributions from covalent bond formation are typically neglected. This approach also requires a ligand preparation step in which the electrophilic warhead is converted to the appropriate reacted form (or multiple different forms, depending on the stereochemistry of the reaction) for subsequent linking. In this context, dummy atoms are often needed to make the linking process technically practicable. Sgrignani *et al.* developed a protocol for covalent docking of boronic acids to serine β -lactamases based on GOLD, imposing the presence of the covalent bond between the boronic warhead and the catalytic serine [Sgrignani *et al.*, 2015]. Focusing on reproduction of the experimentally determined binding mode of 23 boronic AmpC inhibitors, the authors obtained best results with the ChemPLP scoring function and the inclusion of three conserved water molecules. About 80 % of the complexes could be reproduced with an RMSD below 2 Å in the best-ranked cluster (median RMSD 1.04 Å for the entire data set) [Sgrignani *et al.*,

2015]. The optimized protocol was subsequently applied for virtual screening of a library of 1385 commercially available boronic acids as potential AmpC β -lactamase inhibitors [Sgrignani *et al.*, 2016]. Six compounds were selected, experimentally tested and found to be inhibitors of various AmpC β -lactamases in the low micromolar range.

Structure-based virtual screening procedure was also employed in a study to screen the potential inhibitors for OXA-10 ESBL expressing *P. aeruginosa* against the millions of compounds present in the ZINC database [Malathi and Ramaiah, 2016]. It was also utilized to identify novel inhibitors for Penicillin binding protein 2a (PBP2a) of ceftaroline resistant and methicillin-resistant *Staphylococcus aureus* (MRSA) [Lavanya *et al.*, 2016]. Docking has also been used to re-screen through a set of over 70,000 molecules where no specific reversible inhibitors were found among the initial hits, in search of molecules that might be screened at higher concentrations [Babaoglu *et al.*, 2008] (Figure 27). This approach allowed for the identification of novel chemotypes with weak inhibitory activity, and the design of analogues with improved affinity.

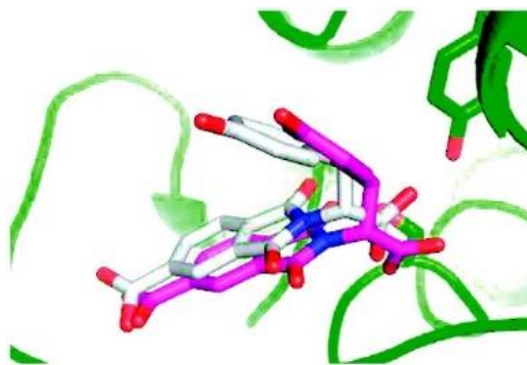


Figure 27. Overlay of the crystal structure (white carbons) overlaid to the initial pose proposed by DOCK (magenta carbons). The RMSD of the heavy atoms between the two poses was 0.9 Å. Image from [Babaoglu *et al.*, 2008].

C) Molecular dynamics simulations:

Atomic-level structures can be extremely helpful in the study of proteins and generate substantial insight about how the biomolecule functions. The atoms in a biomolecule are in constant motion, however, and both molecular function and intermolecular interactions depend on the dynamics of the molecules involved. Unfortunately, watching the motions of individual atoms in a sample and perturbing them in a desired fashion is very difficult, if possible. An attractive alternative is to perform molecular dynamics (MD) simulations, which allows us to work with an atomic-level computer simulation of the relevant biomolecules. Simulations may be used both to interpret experimental results and to propose new hypotheses to guide experimental work.

MD simulations work in the following way. Given the positions of all of the atoms in a biomolecular system (e.g. a protein surrounded by water), one can calculate the force exerted on each atom by all of the other atoms. One can thus use Newton's laws of motion to predict the spatial position of each

atom as a function of time [Karplus and McCammon, 2002]. In particular, one steps through time, repeatedly calculating the forces on each atom and then using those forces to update the position and velocity of each atom. The resulting trajectory is, in essence, a three-dimensional movie that describes the atomic-level configuration of the system at every point during the simulated time interval, which is very difficult with any experimental technique. These simulations can capture a wide variety of important biomolecular processes, including conformational change, ligand binding, and protein folding, revealing the positions of all of the atoms at femtosecond temporal resolution. Importantly, such simulations can also predict how biomolecules will respond—at an atomic level—to perturbations such as mutation, phosphorylation, protonation, or the addition or removal of a ligand.

Second, the simulation conditions are precisely known and can be carefully controlled: the initial conformation of a protein, which ligands are bound to it, whether it has any mutations or post-translational modifications, which other molecules are present in its environment, its protonation state, the temperature, the voltage across a membrane, and so on. By comparing simulations performed under different conditions, one can identify the effects of a wide variety of molecular perturbations.

MD simulations have a number of limitations. The forces are calculated using a model known as a molecular mechanics force field, which is fit to the results of quantum mechanical calculations and, typically, to certain experimental measurements. For example, a typical force field incorporates terms that capture electrostatic (Coulombic) interactions between atoms, spring-like terms that model the preferred length of each covalent bond, and terms capturing several other types of interatomic interactions such as bond angles and dihedral angles. Such force have been improving substantially [Lindorff-Larsen *et al.*, 2012], but they remain inherently approximate, and this should be kept in mind when analyzing simulation results. Another drawback of classical MD simulations is that, unlike with quantum mechanics/molecular mechanics (QM/MM) simulations [Senn and Thiel, 2009], no covalent bonds form or break here.

To ensure numerical stability, the time steps in an MD simulation must be short, typically only a few femtoseconds each. Most of the events of biochemical interest (e.g. functionally important structural changes in proteins) take place on timescales of nanoseconds, microseconds, or longer. A typical simulation thus involves millions or billions of time steps. This fact, combined with the millions of interatomic interactions typically evaluated during a single time step, causes simulations to be very computationally demanding.

Expertise is required in figuring out which questions can be addressed by simulations, designing simulations to address these questions, and interpreting the simulation results, which can be particularly challenging.

Regarding β -lactamases, MD simulations have been used to explore the dynamics of novel β -lactamase variants to try to explain altered phenotypes [Mitchell *et al.*, 2015] (Figure 28), or the interaction between substrates and β -lactamases to explore their influence on the hydrolysis mechanism [Docquier *et al.*, 2009]. The first step involved in MD simulations, energy minimization, has also been used complementarily with molecular docking, in structure-guided inhibitor design attempts [Thakur *et al.*, 2013], or to explore the structural basis of inhibitor binding stereoselectivity [Michaux *et al.*, 2005].

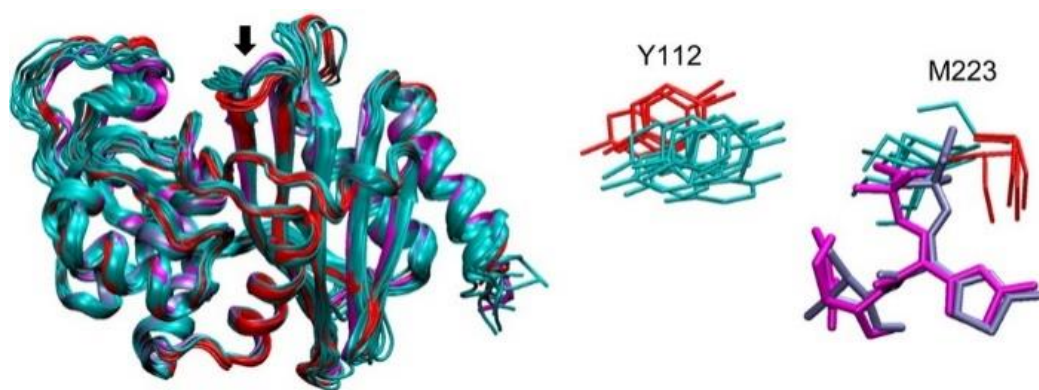


Figure 28. Comparison of conformational diversity in OXA-160 and OXA-24/40. Left panel: representative conformers observed in molecular dynamics simulations for OXA-160 (two clusters shown in cyan and red). The two OXA-160 structures from this study (PDB 4X53 and 4X56, blue and magenta respectively) are included in the alignment (ligands not shown). The $\beta 5$ - $\beta 6$ loop is marked with an arrow. Right panel: Side-chains for the bridge residues Y112 and M223 from the same representative structures. Also shown are ceftazidime (magenta) and aztreonam (blue) from the two OXA-160 X-ray structures after alignment of those structures with the simulation conformers. Authors propose that the red cluster may be the strongest contributor to the increased binding affinity of OXA-160 (OXA-23 P225S mutant) compared to its parental enzyme OXA-23. Image from [Mitchell *et al.*, 2015]

D) HOP software - dynamic water network analysis software:

Proteins exist in aqueous solution. Hydration can be viewed as a description of how the protein disturbs the structure and dynamics of water. Water molecules in the vicinity of proteins are generally seen as either external or internal water molecules. Internal water molecules occupy cavities, exchange on a time-scale of 0.1 to 10 microseconds with bulk water, are almost as conserved as amino acids, and are therefore likely to be important for function. External water molecules tend to be found in protein crevices and are typically not conserved, even between crystal structures of the same protein. The Hop package (Hop software version 0.4.0 alpha 2: <https://github.com/Becksteinlab/hop>) introduce a method to analyse the behaviour of water molecules in molecular dynamics (MD) simulations in terms of graphs [Beckstein *et al.*, 2009]. The graph encodes a simple hopping model. Nodes in the graph correspond to hydration sites, typically defined from the density in computer simulations or observed water sites in crystal structures. Directed edges correspond to transitions ("hops") between sites, with transition rates computed from MD simulations (Figure 29).

Applied to the water-filled cavity of intestinal fatty acid binding protein (I-FABP) in its apo and holo (palmitate-bound) state, this analysis demonstrates how ligand binding influences the well-defined set of hydration sites in and around the protein's cavity. The ligand displaces a number of hydration sites but does not affect others close by. The parameters extracted from the network model allow to model the movement of water molecules with a Markov Chain Monte Carlo model. The graphical construct reproduces the average site occupancy found in the MD simulations and the fluctuations of the occupancy. This approach suggests new types of sampling and analysis that can be applied to extend the range of molecular dynamics models and the role of water in ligand binding.

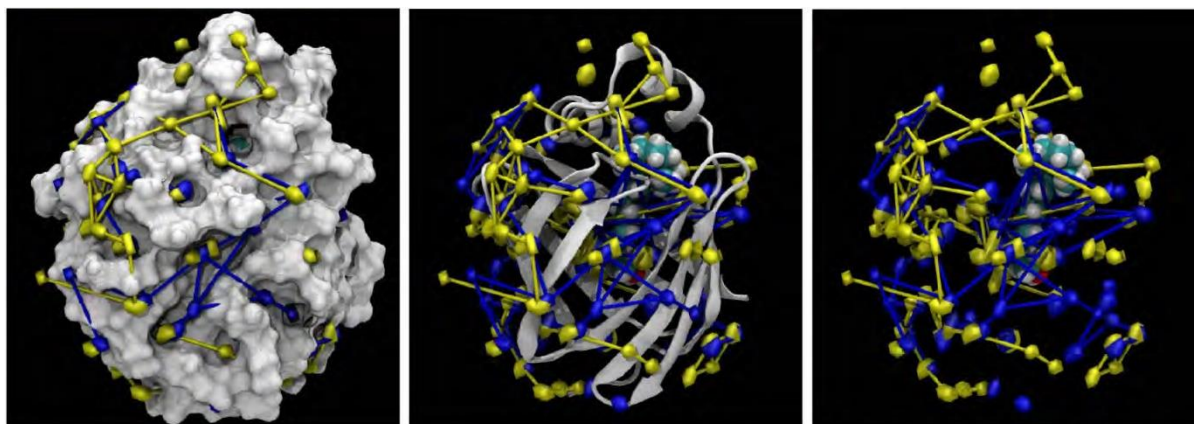


Figure 29. Hopping graph between hydration sites in CRBP II (cellular retinol binding protein II) from 20 ns of MD simulations. Blue: apoenzyme. Yellow: holo complex (bound retinol shown).

In the case of β -lactamases this type of analysis can reveal important factors that may be taken into account for the analysis of ligand binding [Powers *et al.*, 2002] and acyl-enzyme complex hydrolysis rate [Bebrone *et al.*, 2013; Docquier *et al.*, 2009].

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Objectives and scientific approach

Antimicrobial resistance (AMR) is a critical public health threat worldwide, β -lactams are the most used antimicrobials because of their efficacy and safety, but their use leads to emergence and dissemination of resistance against even the latest and most potent ones. Therefore, the development of novel β -lactam drugs, but, more importantly, novel inhibitors that may allow us to continue using the already available ones, is one of the strategies adopted to tackle the AMR issue. *In silico* screening and drug design is a promising strategy to help the rational development of these compounds, but in order to improve the chances of success, and given that these can work as mechanism-based inhibitors, it is important to better understand the structure-function relationship of the target enzymes and also to evaluate and improve the different laboratory and *in silico* techniques that may be applied for the study of targets, screening and development of compounds.

Therefore, the aim of the studies included in this manuscript is:

- to characterize, both biochemically and structurally, naturally occurring and laboratory-made serine- β -lactamase mutants in order to better understand the structure-function relationship of β -lactamases, namely CMY-136, OXA-427, OXA-517, OXA-519, OXA-535, KPC-28, and synthetic variants of OXA-48;
- to use different microbiological techniques, molecular biology and biochemistry techniques, X-ray crystallography and molecular modelling techniques, in order to carry out these studies and to apply them for the future rational design of inhibitors, namely directional cloning, inhibition constant determination, covalent MD simulations, and dynamic water network analysis;
- to develop a database containing information pertinent for the study of β -lactamases, that may be useful for the whole community of microbiologists, namely BLDB.

In order to fulfil these objectives, a complementary approach has been taken, combining different techniques for the study of β -lactamases, namely microbiological techniques such as antimicrobial susceptibility tests, molecular biology techniques such as PCR, cloning and next-generation sequencing, biochemical techniques such as protein purification and kinetic parameters determination, protein crystallization and X-ray crystallography, and molecular modelling techniques such as homology modelling, molecular docking, molecular dynamics simulations, dynamic water network analysis and free energy calculations. The implementation of these techniques should allow us to expand our knowledge of β -lactamases, and to test the suitability of these techniques and improve them for their future implementation in the design of inhibitors.

Publications

Section 1: Characterization of β -lactamases

Antimicrobial resistance poses a threat to public health, which must be faced given the use of antimicrobials is a cornerstone of modern medicine as we know it. The most used antibiotics are β -lactams, and the most common mechanism of resistance to them is the expression of β -lactamases. Therefore, the study of emerging natural β -lactamases, and of laboratory designed mutants, may provide valuable information that might be useful to combat the protection they confer to pathogens, as well as to try to anticipate the direction in which they may evolve given the pressure of antimicrobial therapies in use today.

Novel β -lactamases are detected from clinical isolates based on antimicrobial susceptibility tests such as disk diffusion test or minimal inhibitory concentration tests. They are genotypically characterized by PCR and sequencing, phenotypically by subcloning into cloning plasmids and laboratory strains, biochemically by overexpression and purification and determination of their kinetic parameters against different β -lactams, and structurally by X-ray crystallography and molecular modelling techniques such as molecular docking and molecular dynamics simulations. They are used to provide hypothesis regarding the structural basis of their hydrolytic profile. In the case of OXA-48, mutations were introduced in the β 5- β 6 loop to explore its role in the substrate specificity of OXA-48.

Novel β -lactamases belonging to classes C (CMY-136), D (OXA-427 and OXA-48-like natural and synthetic mutants) and A (KPC-28) were characterized. In the case of CMY-136, results demonstrate how unusual mutations may expand the hydrolytic profile of class C enzymes, by affecting the conformation and stability of conserved loops, in this case the Ω loop. In the case of KPC-28, the results demonstrate how point mutations in this enzyme can alter its hydrolytic profile profoundly, which cannot be revealed by routine molecular detection assays. This enzyme provides an example of how β -lactamases may evolve resistance towards a novel combination therapy such as ceftazidime/avibactam by increasing hydrolysis towards the antibiotic, not actually escaping inhibition by the inhibitor. Molecular docking experiments suggest how mutations producing small conformational changes in the enzyme may ultimately cause large changes in the hydrolytic profile. The characterization of OXA-427 reveals a class D enzyme capable of hydrolysing members of each β -lactam family, posing a potential threat, considering the plasticity of β -lactamases to mutate and increase their hydrolytic efficiency towards a particular substrate. Its structural characterization also reveals peculiar features, such as a novel hydrophobic bridge not present in other β -lactamase structures, that may provide the structural basis to explain its hydrolytic profile. The studies of naturally-occurring and rationally-designed mutants of OXA-48 corroborate how the β 5- β 6 loop is a key feature determining the hydrolytic profile within this family, and also points at how mutations on certain positions within this loop can have higher impact than others. All these studies serve to increase our comprehension of the structural factors governing the functional behaviour of serine- β -lactamases, and at the same time corroborate how β -lactamases are plastic enzymes capable of evolving to change their hydrolytic profile, conferring resistance to drugs they could not hydrolyse before.

Genetic, biochemical and structural characterization of CMY-136 β -lactamase, a peculiar CMY-2 variant

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Running title: *Characterization of CMY-136 beta-lactamase*

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ABSTRACT

With the widespread use and abuse of antibiotics for the past decades, antimicrobial resistance poses a serious threat to public health nowadays. β -Lactams are the most used antibiotics, and β -lactamases the most widespread resistance mechanism. Class C β -lactamases, also known as cephalosporinases, usually do not hydrolyse the latest and most potent β -lactams, expanded spectrum cephalosporins and carbapenems. However, the recent emergence of extended-spectrum AmpC cephalosporinases, their resistance to inhibition by classic β -lactamase inhibitors, and the fact that they can contribute to carbapenem resistance when paired with impermeability mechanisms, means that these enzymes may still prove worrisome in the future. Here we report and characterize the CMY-136 β -lactamase, a Y221H point mutant derivative of CMY-2. CMY-136 confers an increased level of resistance to ticarcillin, cefuroxime, cefotaxime and ceftolozane/tazobactam. It is also capable of hydrolysing ticarcillin and cloxacillin, which act as inhibitors of CMY-2. X-ray crystallography and modelling experiments suggest that the hydrolytic profile alterations seem to be the result of an increased flexibility and altered conformation of the Ω -loop, caused by the Y221H mutation.

INTRODUCTION

CMY-2-like enzymes are plasmidic class C β -lactamases that originated from the chromosomally-encoded AmpC of *Citrobacter freundii*¹. They can be found in humans, feedstock animals, and pets². CMY-2 is the most widespread plasmid-mediated AmpC (pAmpC) β -lactamase, which can be illustrated by the CMY-2-like enzymes reported to date, which amount to over 140³. They are also known as cephamycinases, because of their potential to hydrolyse these substrates, but they're also

active on penicillins and first generation cephalosporins, and fairly resistant to inhibition by classical β -lactamase inhibitors (clavulanic acid, tazobactam and sulbactam)¹. They are less active against third or fourth generation cephalosporins, and almost completely inactive against carbapenems. Lately however, several class C enzymes with extended-spectrum, termed extended-spectrum AmpC cephalosporinases (ESACs)⁴, have been reported, among them members of CMY-2-like family. Many of these enzymes show increased activity against third generation cephalosporins such as ceftazidime or cefotaxime, and even cefepime, a fourth-generation cephalosporin. When paired with impermeability mechanisms, CMY-2 may even mediate carbapenem resistance⁵. These enzymes are of clinical importance, and their study may prove important not only in understanding how their hydrolysis profile might evolve, but also to gain insights into their structure-function relationship, which in turn may aid in the development of new inhibitors, not only for class C but also for other classes of β -lactamases. We report here a novel CMY-2-like β -lactamase, CMY-136, we characterize it both biochemically and structurally, and provide the possible structural basis behind its hydrolysis profile differences when compared to CMY-2.

RESULTS

Isolation of *bla*_{CMY-136} gene

E. coli EC13 clinical isolate, identified from a urinary tract infection, displayed an unusual cephalosporinase phenotype⁶. This isolate was resistant to ticarcillin, susceptible to cefoxitin, and showed increased susceptibility to inhibition by clavulanic acid, that was reversed on cloxacillin containing plates, suggesting a class C enzyme. PCR was positive for CMY-2, and sequencing revealed a novel CMY-2-like variant, which was assigned the name CMY-136, differing by a single amino acid substitution, Y221H, in a highly conserved position in class C β -lactamases³.

Phenotypical characterization of CMY-136

The antimicrobial susceptibility profile conferred by CMY-136 was compared to that of CMY-2 by transforming *E. coli* TOP10 cells with a pTOPO plasmid harbouring either blaCMY-2 or blaCMY-136 genes and performing disk diffusion susceptibility tests and MICs assays. CMY-2 and CMY-136 show similar resistance profiles, they both confer resistance to penicillins and cephalosporins, and show susceptibility to cefepime and carbapenems. Based on the disk diffusion test (data not shown), when compared to CMY-2, CMY-136 confers increased resistance to ticarcillin and cefotaxime, low level resistance to cefoxitin, and shows increased susceptibility to inhibition by clavulanic acid. The MICs show that when compared to CMY-2, CMY-136 confers to *E. coli* decreased resistance to piperacillin and cefoxitin, and an increased level of resistance to ticarcillin, cefuroxime and cefotaxime (Table 1). According to the cut-off values of EUCAST, *E. coli* TOP10 expressing CMY-136 is also resistant to ceftolozane/tazobactam, whereas under the same conditions CMY-2 confers no resistance to it. Similarly, CMY-136 also shows reduced susceptibility to ceftazidime/avibactam.

Table 1 MICs for *E. coli* TOP10 pTOPO-CMY-2 and *E. coli* TOP10 pTOPO-CMY-136.

B-lactam	<i>E. coli</i> TOP10 pTOPO-CMY-2	<i>E. coli</i> TOP10 pTOPO-CMY-136
Amoxicillin	>256	>256
Amoxicillin + CLA ^a	32	24
Ticarcillin	32	>256
Temocillin	24	24
Piperacillin	24	12
Piperacillin + TAZ ^b	4	2
Cefoxitin	>256	16
Cefuroxime	24	>256
Cefotaxime	8	>32
Ceftazidime	24	24
Ceftazidime + AVI ^b	0.38	0.75
Cefepime	0.125	0.094
Ceftolozane + TAZ ^b	0.5	2.5
Imipenem	0.25	0.19
Meropenem	0.032	0.023
Ertapenem	0.012	0.012
Aztreonam	2	3

^aCLA: clavulanic acid

^bTAZ: tazobactam

^cAVI: avibactam

Genetic support of bla_{CMY-136} gene

Kieser⁷ extracted DNA from *E. coli* EC13 was electroporated into electrocompetent *E. coli* TOP10 cells and plated on ampicillin-containing plates (100 mg/L). Several colonies that grew on ampicillin were confirmed by PCR and sequencing to be bla_{CMY-136} positive. Kieser DNA of *E. coli* EC13 and of electrotransformed *E. coli* TOP10 cells run on a 0.7% agarose gel revealed a single plasmid, pCMY-136 of ca. 90 Kbp in both isolates. Whole genome sequencing using Illumina technology of *E. coli* TOP10 (pCMY-136) revealed a 90844 bp plasmid in size, that belonged to the IncI1 group, and carried a single antibiotic resistance gene, bla_{CMY-136}. BLAST search showed the closest deposited plasmid sequence to be the bla_{CMY-2} carrying plasmid pCVM22462 (accession number [CP009566.1](#))⁸, isolated from a *Salmonella enterica* strain. It is 99.98% identical to pCMY-136, excluding a 3,888-bp fragment that is absent in pCMY-136. This deletion is located 1,965-bp upstream of bla_{CMY-136}/bla_{CMY-2} genes. This 3,888-bp fragment contains 3 hypothetical protein ORFs and occurred contiguously downstream of an IS_{I294}-like insertion sequence present on both plasmids.

Purification and biochemical characterization of CMY-136

CMY-136 and CMY-2 were overexpressed and purified by Immobilized Metal Affinity Chromatography (IMAC), and purity of both enzymes was confirmed by SDS-PAGE, where a single band of ca. 41 kDa was observed. Steady-state kinetic parameters were determined to compare the catalytic activity of CMY-136 to that of CMY-2 against several β -lactams (Table 2). CMY-136 showed around a 10-fold increase in K_m for most β -lactams assayed, with however, a few exceptions: (i) cefalotin (CEF), which showed a slightly higher affinity for CMY-136 than CMY-2, although other authors have reported a lower K_m for CMY-2 against CEF^{9,10,11}; (ii)

cefoxitin (FOX), which showed a practically identical K_m for both enzymes; (iii) β -lactams with a very high affinity for CMY-2 and a bulky, non-linear, R1 substituent, namely cefuroxime (CXM), cefotaxime (CTX) and ceftazidime (CAZ), which show a 10^3 - 10^4 -fold increase in K_m for CMY-136; (iv) other β -lactams, namely ticarcillin (TIC) and cloxacillin (CLX), for which CMY-2 shows high affinity and no hydrolysis unlike CMY-136 that does hydrolyze them.

CMY-136 showed a decreased k_{cat} for most of the preferred substrates of CMY-2, namely amino-penicillins and first generation cephalosporins, and also cefoxitin. However, it also shows an increased k_{cat} for cefepime and ceftolozane, and a remarkably increased k_{cat} (10^2 - 10^3 -fold) for those β -lactams for which the most dramatic K_m increase was also seen, CAZ, CTX, CXM. CMY-136 is also capable of hydrolysing cloxacillin and ticarcillin, which are not hydrolysed by CMY-2. In terms of their catalytic efficiency, all these translate into CMY-136 showing a lower efficiency than CMY-2 for most β -lactams tested, except for those not hydrolysed by CMY-2, TIC and CLX, and also ceftolozane.

IC50 for aztreonam, tazobactam, and clavulanic acid were 0.11 μ M, 5.5 μ M and 137 μ M, respectively, making CMY-136 similarly susceptible to inhibition by tazobactam as CMY-2¹².

Table 2 Steady-state kinetic parameters of β -lactamases CMY-136 and CMY-2.

Substrate	K_m (μ M)		k_{cat} (s^{-1})		k_{cat}/K_m ($mM^{-1} s^{-1}$)	
	CMY-2	CMY-136	CMY-2	CMY-136	CMY-2	CMY-136
Benzylpenicillin ^b	0.6	10.1	24	7.83	40000	775
Cloxacillin ^b	0.0002	543	N.H.	0.47	N.H.	0.9
Ampicillin ^c	0.16	3.5	0.55	0.43	3437	122
Ticarcillin	0.03	1.7	N.H.	0.15	N.H.	86
Cefaloridine ^a	130	>1000	421	169	3240	110
Cefalotin ^a	90	35	217	21.65	2400	618
Cefoxitin ^b	0.15	0.2	0.35	0.02	2333	111
Cefuroxime ^b	0.005	21.6	0.014	9.42	2800	436
Cefotaxime ^a	0.001	20	0.007	4.71	7000	236
Ceftazidime ^a	0.15	>1000	0.005	6.26	33	2.7
Cefepime	412	>1000	0.37	1.79	0.9	0.5
Ceftolozane	200	>1000	0.69	13.27	3.5	8.4

Imipenem ^a	0.8	10.9	0.03	0.01	38	1.2
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N.H: no hydrolysis
^a taken from ^{13,5}
^b taken from ⁹
^c taken from ¹¹

CMY-136 crystallization and X-ray crystallography

CMY-136 crystallized in 0.1 M HEPES pH 7.5; 20% w/v PEG4000; 10% isopropanol. The structure was determined by molecular replacement using the deposited CMY-2 structure (PDB: 1ZC2) as a template, and the model was refined to 1.60 Å (Table 3). The asymmetric unit contains two protein chains, A and B, showing no significant conformational differences between them. They have been modelled with 359 residues each, with clear electron density observed for all regions of the protein. Several ordered water molecules have been modelled in the structure as well as phosphate anions and isopropyl alcohol molecules, from the crystallization condition. Both chains present the typical class C fold with an α -helical region and a mixed α -helix/ β -sheet region. 99% of all residues are inside the favoured regions of the Ramachandran plot, and 1% in the allowed regions.

Table 3 Crystallography data collection and refinement statistics.

Data collection	
Space group	P 1 2 ₁ 1
Cell dimensions	
a, b, c (Å)	60.58, 58.09, 100.08
α , β , γ (°)	90.00, 89.97, 90.00
Resolution (Å)	16.1-1.60
Rmerge	0.092
I/ σ (I)	3.09 (at 1.6Å)
Completeness (%)	94.2
Redundancy	4.2
Refinement	
Resolution range (Å)	16.10-1.60
No. unique reflections	87,503
R _{work} /R _{free}	23.4%/26.4%
No. non-hydrogen atoms	

Protein	5,642
Water	396
Ligand/Ions	67
Total	6105
Average B, all atoms (Å ²)	19.8
Protein	19.4
Water	25.2
Ligand/Ions	29.6
Root mean squared deviations	
Bond lengths (Å)	0.01
Bond angles (°)	1.04

CMY-136 shows the same overall conformation as CMY-2, and 3D alignment reveals a C α RMSD of 0.465-0.486 Å between them. Several structural differences can be found when comparing CMY-136 (Fig. 1) to CMY-2: (i) A phosphate molecule is positioned inside the active site of both CMY-136 chains, with one of its oxygens buried inside the oxyanion hole. CMY-2 was crystallized with a citrate molecule inside the active site; (ii) The conformation adopted by the TYR221 in CMY-2, which interacts with the backbone of the Ω -loop at SER212 O and GLN215 O via two bridging waters, is different from the conformation found in CMY-136 for HIS221, whose sidechain invades the active site cavity, hydrogen bonding the aforementioned phosphate oxygen atom. The TYR221 residue and its conformation are highly conserved in all class C enzymes reported to date (with the exception of PDC-85) and in all class C crystallographic structures deposited in the PDB that have a consensus length Ω -loop; (iii) In turn, the sidechain of ASP217 turns towards the interior of the Ω -loop to partially occupy the space left by the conformational change of the Y221H mutation; (iv) The conformation adopted by the backbone of the Ω -loop residues ARG204 to GLN215 in CMY-136 is different from the one found in CMY-2, that is also highly conserved in deposited class C structures with a consensus length loop, which translates into a backbone atoms RMSD between said residues in both crystal structures of 2.176 - 2.219 Å, a 4.56 - 4.68 Å displacement in the position of the C α of VAL211, and the loss of the hydrogen bonds between GLU61 O ϵ and VAL211 N (Fig. 2) and between VAL209 O and GLY202 N (Fig. 2B). Two water molecules, w502

and w504, occupy the usual space of residues V209-V211 backbone, and partially replace those lost hydrogen bonds (Fig. 2B); (v) The configuration of HIS210 sidechain is shifted from being positioned between, and interacting via pi-pi stacking with, TYR199 and TRP201 in CMY-2 – another highly conserved feature of class C structures, that fill this space with either HIS210 or ARG210 sidechains - to not directly interacting with any other residue, and hydrogen bonding w502 (Fig. 2B). The space between TYR199 and TRP201 in the CMY-136 structure is filled by LYS290 from another CMY-136 chain in the neighbouring asymmetric unit in the crystal structure; (vi) The conformation of the active site cavity residues and the hydrogen bond network between them is highly conserved, with the exception of TYR150, which is slightly shifted towards the phosphate anchored in the active site, with a 2.7-3.0 Å displacement of its hydroxyl oxygen atom compared to TYR150 of the CMY-2 structure. A water molecule can be found occupying this position, and TYR150 is still hydrogen bonding LYS67, and now also hydrogen bonds with the phosphate molecule and possibly ASN152, but no longer interacts with LYS315.

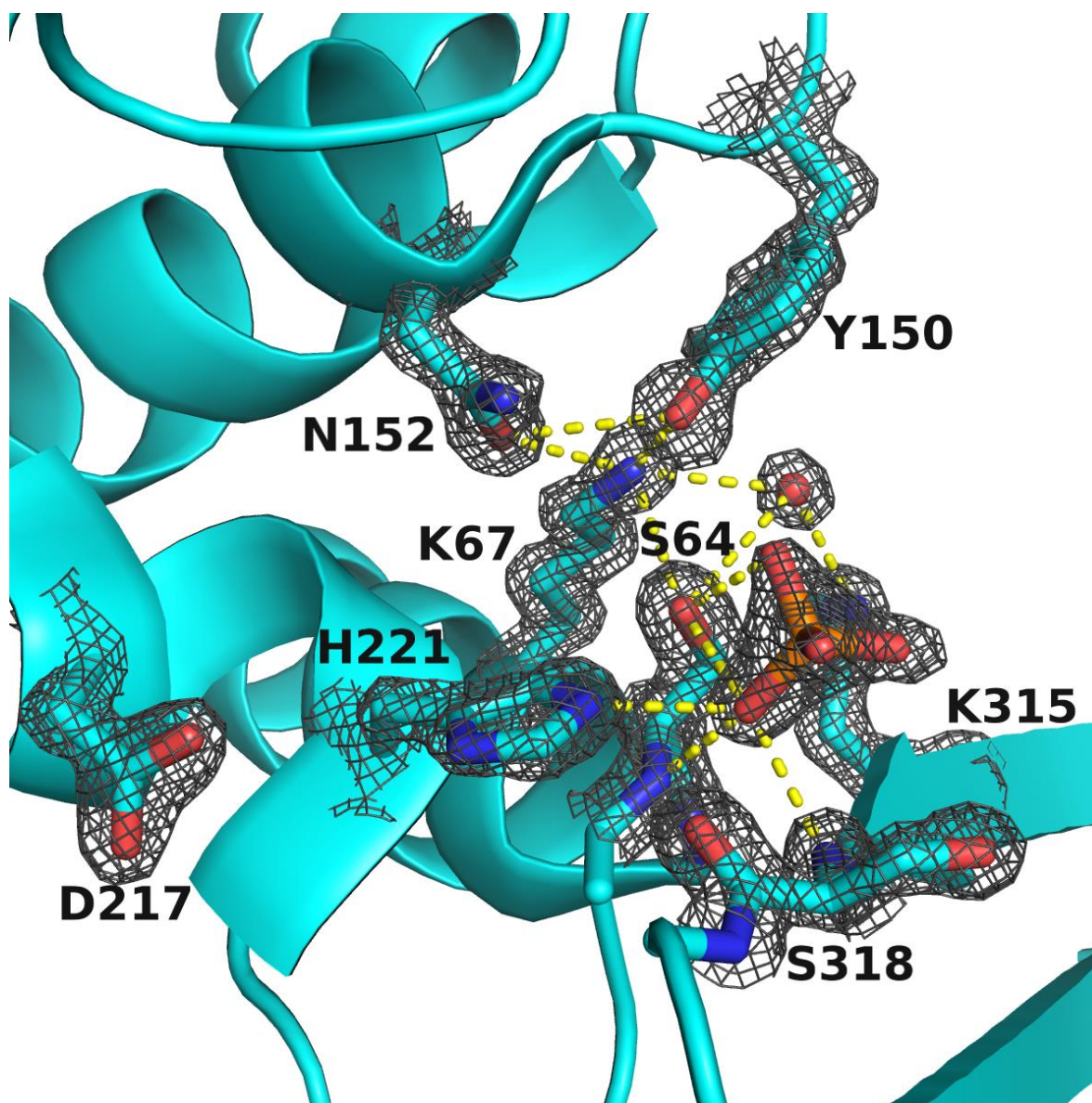


Fig. 1 Crystal structure of CMY-136. Active site cavity of CMY-136, with a phosphate molecule interacting with the oxyanion hole and other important residues. Note the conformation of the H221 sidechain, the position shift of Y150, and the water molecule occupying its hydroxyl usual position. Hydrogen bonds are depicted as dashed yellow lines.

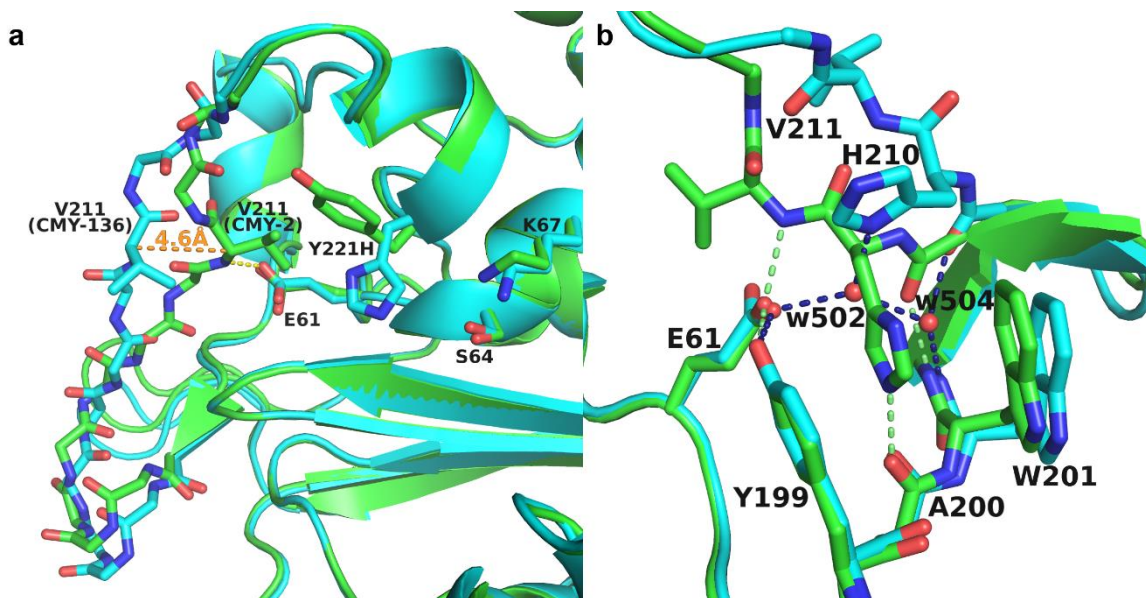


Fig. 2 Comparison of the Ω -loop of CMY-136 and CMY-2. a) Front view, the conformational change in the Ω -loop for CMY-136 widens the active site cavity, displacing V211 C α about 4.6 Å, and breaking the hydrogen bond with E61. Hydrogen bonds are depicted as dashed yellow lines. Displacement depicted as dashed orange lines. b) Side view, the conformational change also displaces H210 sidechain from its usual position interacting with residues 199-201. Hydrogen bonds are displayed as colored dashed lines according to model colors.

Covalent docking of β -lactams on CMY-2 and comparison with CMY-136

As no crystal structure of CMY-2 co-crystallized with β -lactams is available, covalent docking of the open β -lactams' acylating structure was performed, for both CMY-2 and CMY-136, to explore what conformation the acyl-enzyme complexes could adopt after initial hydrolysis, that may explain the rise in K_m observed for CMY-136 compared to CMY-2. Docking conformations on CMY-2 for ceftazidime and cefoxitin (Fig. 3) show that β -lactams fill the binding site cavity and establish the expected conserved interactions (R1 and R2 sidechains positioned towards the corresponding pockets, R1 amide hydrogen bond interactions with S318 O and N152 and Q120 sidechains, β -lactam carbonyl oxygen buried in the oxyanion hole). Ceftazidime shows a conformation similar to

that observed in the AmpC-ceftazidime complex (PDB 1IEL). The R1 sidechain points towards the omega loop, with the aminothiazole ring contacting Y221 and V211, and the dimethyl carboxyl branch facing away, towards the solvent, and hydrogen bonding S343. The amide group of the R1 sidechain is hydrogen bonding the sidechains of N152 and Q120, and also the backbone of S318. The β -lactam oxygen is buried in the oxyanion hole hydrogen bonding the backbone of SER318 and the catalytic S64. The conserved C3/C4 carboxylate is interacting with K315. Similar interactions are observed in the docking complex of cefoxitin with CMY-2, although because of the conformation adopted by the thiophene ring contacting Y221 and V211, and the presence of the 7 α -methoxy group hydrogen bonding N152, the R1 sidechain is slightly displaced from its usual position towards the solvent, and the R1 amide group only conserves the hydrogen bond with the backbone of S318. Similar binding modes and interactions are observed for the other β -lactams that were covalently docked on CMY-2 (data not shown).

Superposition of the CMY-136 structure on the docking results on CMY-2 (Fig. 4) illustrates how the conformation of H221 in CMY-136 represents a clear steric impediment for the binding of the β -lactams in the active site. There are numerous clashes between the sidechain of H221 and the R1 substituent of all β -lactams, with distances between overlapping atoms ranging from 0.8 to 2.7 Å, depicted in the figures by thin orange full lines. These results would explain the K_m increase observed for CMY-136 for almost all β -lactams.

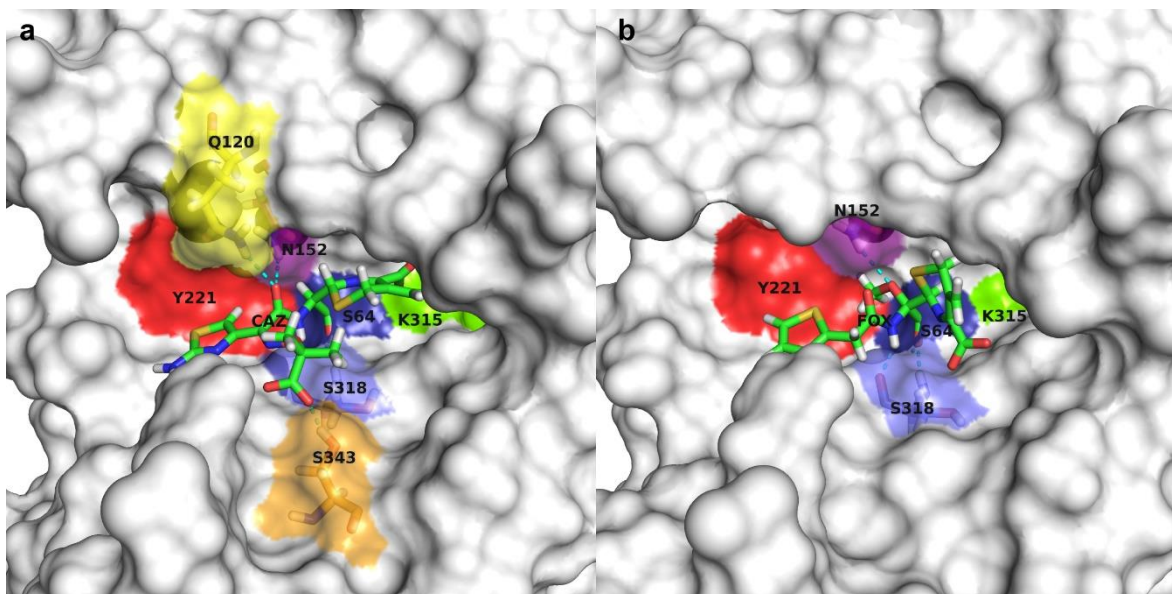


Fig. 3. Docking conformations of CAZ and FOX on CMY-2. Covalent docking of (a) CAZ and (b) FOX on CMY-2. Stick representation of the β -lactams docked inside the active site of CMY-2, represented as white or coloured surface. Y221 forming the active site wall in CMY-2 is colored red. Catalytic S64 is colored dark blue. Other relevant interacting residues are represented in different colors and transparent surface to better perceive the hydrogen bonds (cyan dashed lines): Q120 (yellow), N152 (purple), K315 (green), S318 (light blue), S343 (orange).

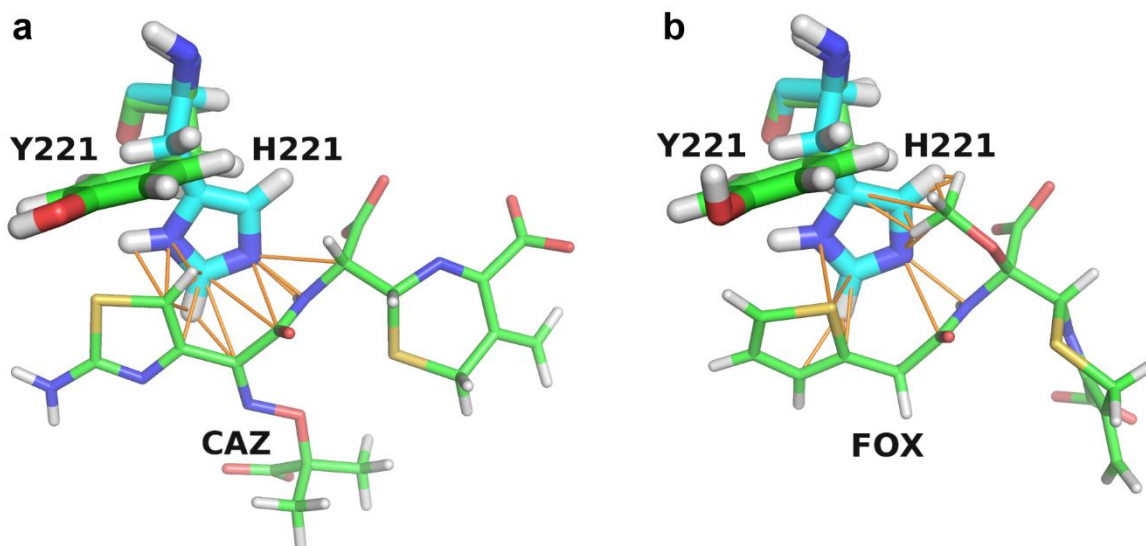


Fig. 4. Steric impediment for β -lactam binding in CMY-136. Superposition of CMY-136 on the covalent docking complexes of (a) CAZ-CMY-2 and (b) FOX-CMY-2. Only Y221 (CMY-2) and

H221 (CMY-136) are shown for clarity. Thin orange lines represent potential clashes between H221 and the canonical β -lactam conformation in the acyl-enzyme complex with CMY-2. The distance between such overlapped atoms ranges between 0.8 and 2.7 Å. The conformation adopted by H221 in CMY-136 represents a clear steric impediment for the binding of β -lactams.

Molecular dynamics simulations of CMY-2 and CMY-136

To investigate the role of the Y221 mutation on the dynamics of the protein structure, molecular dynamics (MD) simulations were performed with four different systems: CMY-2, CMY-136, CMY-136 with the Ω -loop modelled on the CMY-2 structure, and CMY-2 with the Ω -loop modelled on the CMY-136 structure. Root-mean-square deviation (RMSD) analysis of the trajectories reveals that the simulations reached stability within 2 ns and remained stable after that. Root-mean-square fluctuation (RMSF) analysis of C α atoms in CMY-2 and CMY-136, expressed as B-factors, reveals some differences between them, regarding the flexibility of several parts of the structure (Fig. 5A). CMY-136 displays increased flexibility in the Ω -loop and H7 helix, from R204 up to H221, except for a short segment in the middle. An increased flexibility can also be found for part of the Q120 loop, part of H10 and H11 helices, and the C terminus of B3 strand, S318 and T319. Other loops around CMY-136 also have increased flexibility compared to CMY-2, to a lesser extent. There are also segments of decreased flexibility towards the exterior of the structure, contiguous to the previously described regions, the N-terminus of H10 helix and its preceding loop, C-terminus of H11 helix, H4 helix, G320, part of helices H5 and H6, directly behind H4 helix in the tertiary structure, and also S343, next to the C-terminus of strand B3. It should be noted that R349, in the middle of H11, also shows decreased flexibility. Regions with increased flexibility can be found surrounding and forming the walls of the active site cavity, with regions of decreased flexibility further away (Fig. 5B).

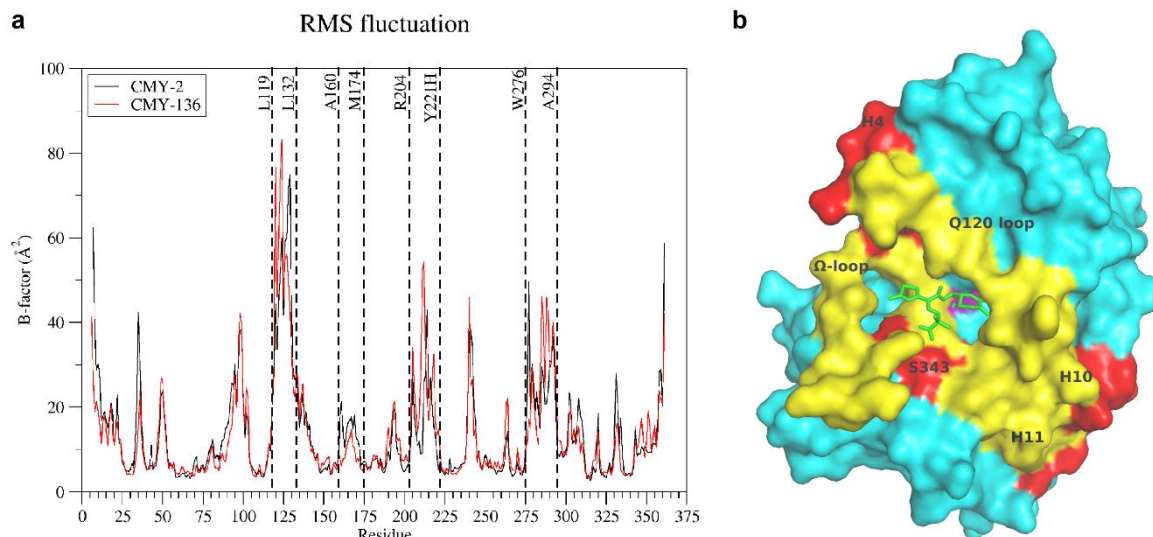


Fig. 5 Altered flexibility of CMY-136 Structure. a) CMY-2 and CMY-136 RMSF analysis, expressed as C α B-factors. Compared to CMY-2, several regions displayed increased or decreased flexibility in the CMY-136 structure during MD simulation: part of the Ω -loop (R204-Y221), Q120 loop (P118-R126), C-terminus of B3 strand (S318-G320), helices H4 (D127-L131), H5 (A160-G167), H6 (S169-M174), H10 (P277-A295), H11 (S343-Q361). b) Surface representation of CMY-136 in the acyl-enzyme complex with CAZ, which is represented as green sticks. The surface of S64 is colored in purple. The surface of regions with increased flexibility, determined by C α B-factors, are colored in yellow, and those with decreased flexibility in red.

The MD simulations also demonstrate how the sidechain of Y221 in CMY-2 remains constantly in the same conformation, pointing outwards of S64, with its hydroxyl hydrogen bonding with the backbone of the Ω -loop directly or through bridging waters (Fig. 6). In contrast, H221 in CMY-136 displays much more flexibility, adopting several conformations in the active site throughout the MD simulation (Fig. 7 and 8). Other parts of the Ω -loop also show increased sidechain flexibility (Fig. 8), namely H210-S212 and Y199-W201, which normally interact closely with each other and with E61.

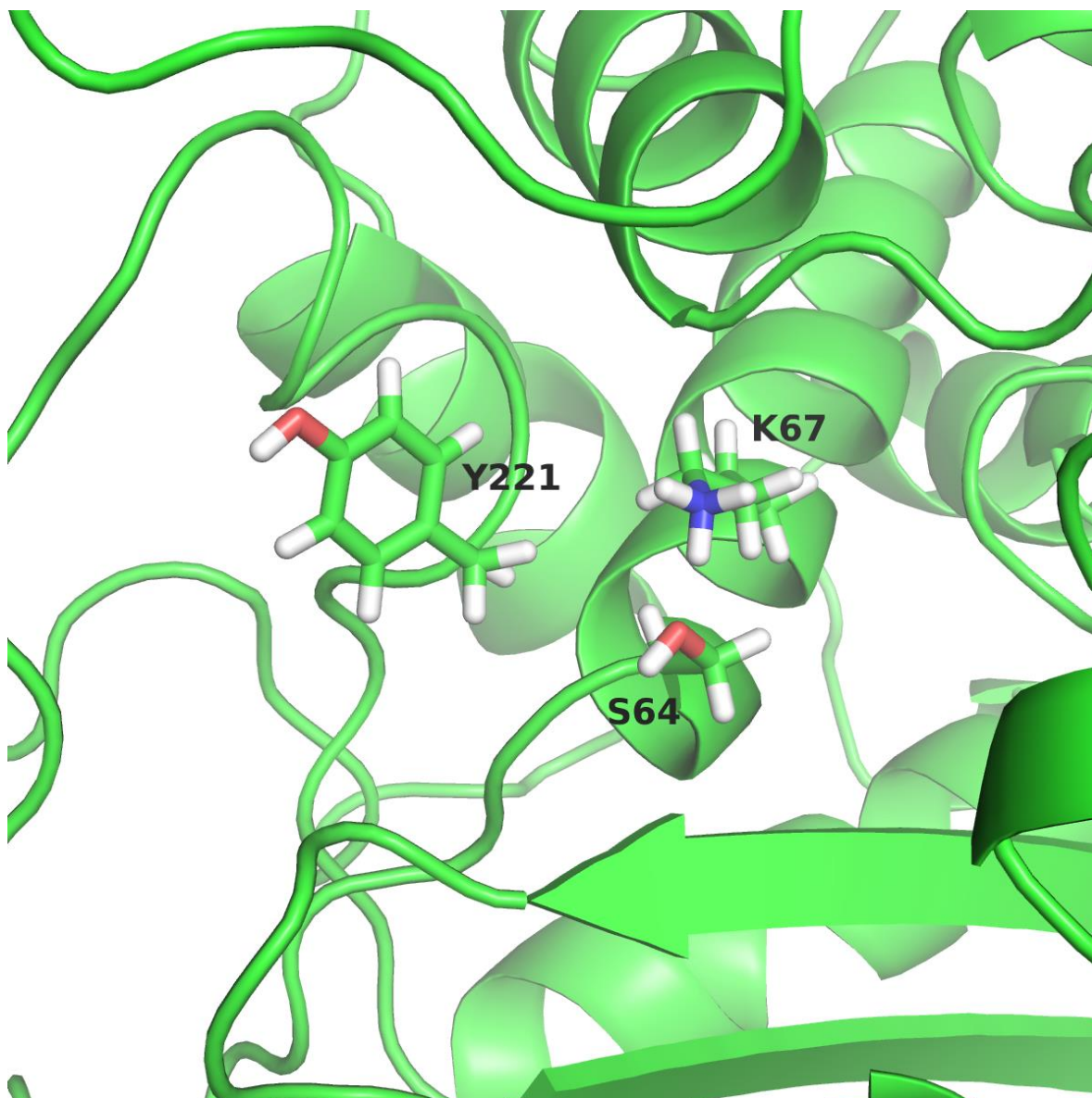


Fig. 6 Flexibility of TYR 221 in CMY-2. Snapshot from the CMY-2 MD simulation depicting the only conformation of Y221 observed throughout the 10 ns simulation.

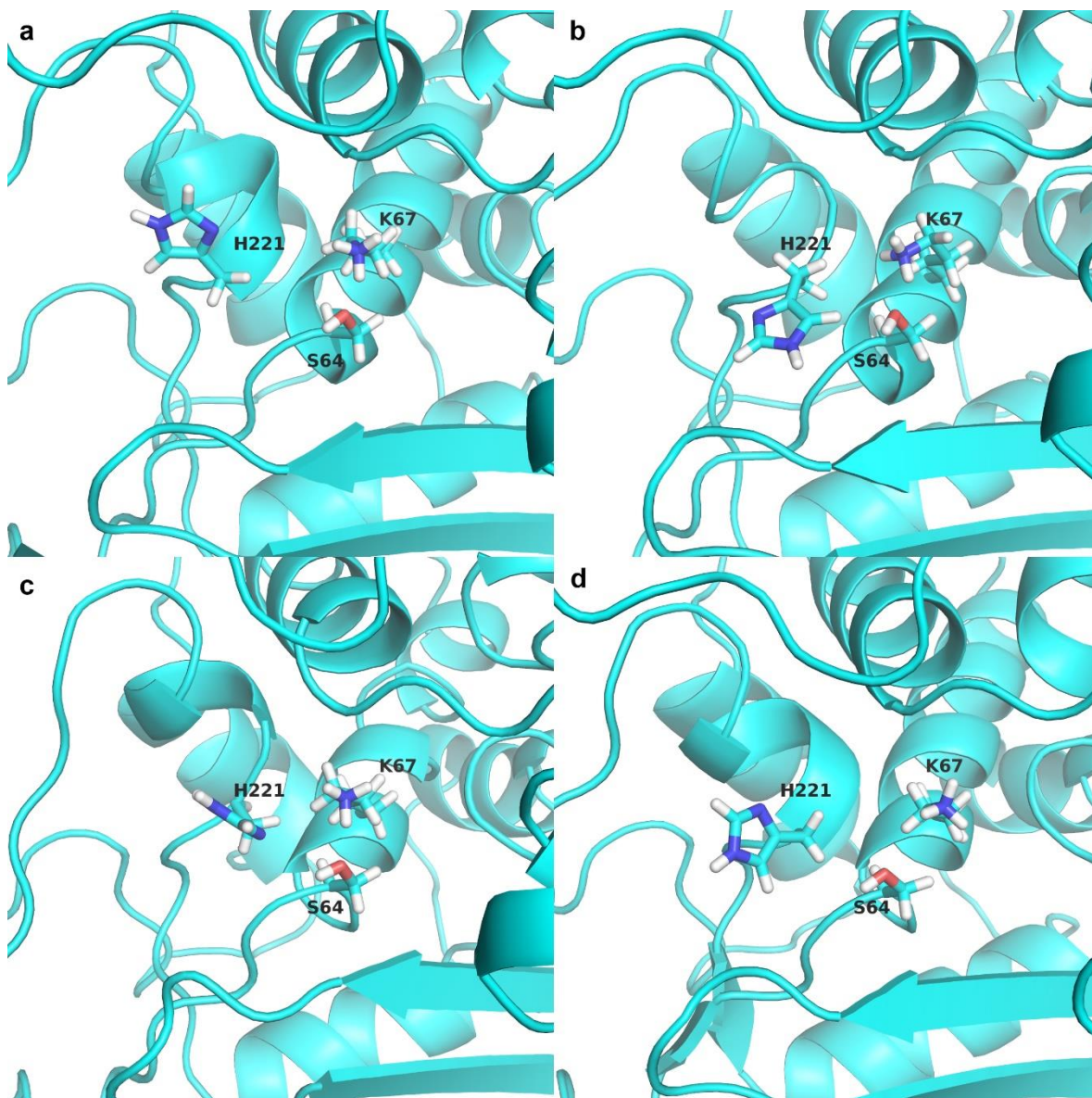


Fig. 7 Flexibility of H221 in CMY-136. Four snapshots of the CMY-136 MD simulation depicting several conformations adopted by H221 throughout the 10 ns simulation.

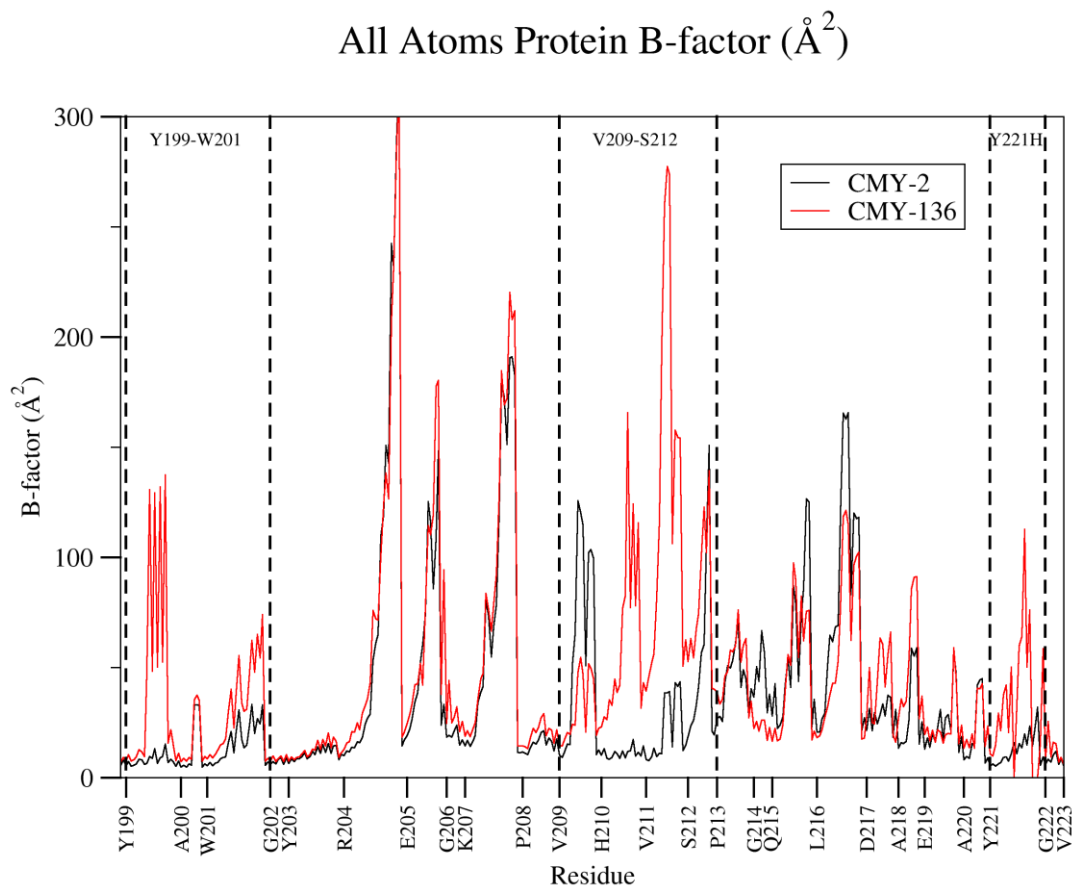


Fig. 8 Flexibility of Ω -loop in CMY-136 vs CMY-2. Individual atom B-factors of CMY-136 and CMY-2 Ω -loops. Note the increased flexibility of residue 221 in CMY-136, and also of regions H210-S212 and Y199-W201, which interact in CMY-2 as shown in Fig. 2.

This same H221 flexibility can be observed for the simulation of the CMY-136 model with the Ω -loop, H7 and H221 sidechain starting with the same conformation as the CMY-2 crystal structure. In contrast, for the CMY-2 model with the Ω -loop, H7 and Y221 sidechain starting with the same conformation as the CMY-136 crystal structure, the native conformation of Y221 is not reached. Snapshots of these four simulations were extracted every 1 ns and fitted on the CMY-2 or CMY-136 crystal structure as references. Ω -loop C α RMSD between the snapshot and the reference was determined, to assess conformational differences (Fig. 9). The analysis shows that both CMY-136 simulations, starting with a Ω -loop, H7 and H221 conformation as that observed in either the CMY-

136 crystal structure or the CMY-2 crystal structure, present a lower RMSD compared to CMY-136 than to CMY-2 crystal structures towards the end of the simulation. Similarly, for the CMY-2 simulation starting from the CMY-2 crystal structure conformation, some fluctuations can be observed during the simulation but finally the RMSD is also lower for its own native crystal structure than for that of the other enzyme, and in the same magnitudes as observed for CMY-136. In contrast, in the simulation of CMY-2 starting with the Ω -loop, H7 and Y221 conformation of the CMY-136 loop does not converge to the CMY-2 crystal structure conformation and remains closer to the CMY-136 crystal structure conformation. As mentioned earlier, for this simulation, Y221 does not acquire the native conformation observed in all class C structures.

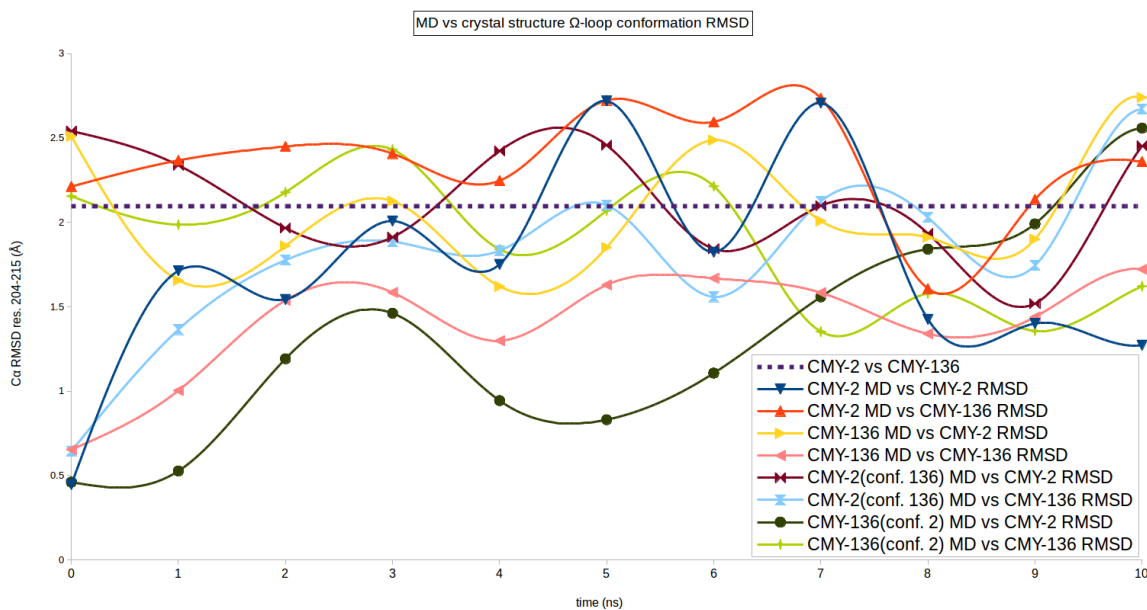


Fig. 9. Ω -loop trajectories RMSD analysis. Ω -loop C α RMSD analysis of the MD trajectories compared to CMY-2 and CMY-136 crystal structures. RMSD was calculated between crystal structure Ω -loops, and MD simulation snapshots taken every 1 ns. Regardless of starting conformation, CMY-136 structures tend to converge to a conformation closer to that of CMY-136 crystal structure, and far from that of CMY-2 crystal structure. For CMY-2 simulations, when starting from the native structure conformation, Ω -loop conformation is maintained closer to it than

to that of CMY-136 Ω -loop crystal structure. When starting from a CMY-136 Ω -loop conformation, however, CMY-2 Ω -loop conformation does not converge towards its own Ω -loop crystal structure conformation. Ω -loop RMSD between both crystal structures is represented by the purple dashed line.

DISCUSSION

We report here a novel β -lactamase belonging to the CMY-2 family, with an unusual mutation, Y221H, in a highly conserved position in class C β -lactamases. Analysis of the plasmid harbouring *bla*_{CMY-136} suggests it may have originated by mutation deriving from the *bla*_{CMY-2} harbouring plasmid pCVM22462. It is noteworthy that the 3888bp fragment absent from pCMY-136 but present in pCMY-2 occurs directly adjacent to IS1294, which may suggest its involvement in the deletion of this fragment¹⁴.

The biochemical characterization of CMY-136 suggests it to be less efficient than CMY-2 in hydrolysing most β -lactams, the exception being ceftolozane, and also those not hydrolysed by the latter, cloxacillin and ticarcillin. The results for these three β -lactams corroborate what can be observed in the MIC assays. However, other significant differences in MICs for CMY-136 when compared to CMY-2 (increased MIC for cefotaxime and cefuroxime, and decreased MIC for cefoxitin) are not reflected in the catalytic efficiencies determined. In this regard, it has been previously suggested that for class C enzymes, k_{cat} may better represent the degree of resistance towards β -lactams^{15,16}. Therefore, the comparison of k_{cat} may explain better what is observed for the MICs. A 10-fold increase in k_{cat} , or bigger, is observed for CMY-136 for cloxacillin, ticarcillin, cefuroxime, cefotaxime, ceftazidime, and ceftolozane, and a 10-fold decrease for cefalotin and cefoxitin. This corresponds well with observed MIC results, except for cloxacillin and cefalotin, for which MICs were not determined, and ceftazidime, which showed no difference in MIC between CMY-136 and CMY-2.

These results for CMY-136 are consistent with previously published results for similar cases. PDC-85, a variant of the chromosomally-encoded AmpC of *Pseudomonas aeruginosa*, is the only other class C enzyme presenting a Y221H mutation¹⁷. Similarly to CMY-136, the PDC-85 variant showed an increase in MIC for ticarcillin and ceftolozane/tazobactam, and a decreased susceptibility to inhibition by cloxacillin, probably related to CMY-136 being capable of hydrolysing cloxacillin. In a different study, it was observed that the *E. coli* AmpC Y221G laboratory mutant showed an increased K_m and k_{cat} for cefotaxime, while at the same time significantly losing activity on cefalotin¹⁶. The authors evidenced that this mutation decreases the enzyme thermal stability, increase flexibility, and expand the active site cavity, and attribute the increase in cefotaxime hydrolysis to these effects, allowing for a better accommodation of the acyl-enzyme complex into a catalytically competent conformation for de-acylation. For cefalotin, they propose the now missing stabilizing interactions between the substrate and the Y221G mutation to be responsible for the loss of hydrolysis.

The crystal structure of CMY-136 shows the same conformation as that of CMY-2, except for those differences described in the Results section. Just as for the Y221G AmpC mutant study, in CMY-136 the space no longer occupied by Y221 is partially occupied by D217, which turns towards the active site cavity. Another possible common feature is the expansion of the active site cavity, as the CMY-136 Ω -loop conformation displaces the V211 C α from its usual position in class C structures by about 4.6Å. It is unclear whether the changes observed in the CMY-136 conformation are the result of the crystal configuration and interactions between neighbouring chains in it, the crystallization with a phosphate molecule in the active site, the consequences of the Y221H mutation, or a combination of them.

In order to investigate the difference in affinity for β -lactams between CMY-2 and CMY-136, covalent docking of β -lactams in the CMY-2 active site was performed, to evidence the conformations adopted by the acyl-enzyme complex and to compare them with the CMY-136 structure. As shown in Fig. 4, the acyl-enzyme complexes for CMY-2 adopt conformations similar

to those observed in other structures of class C enzymes in complex with substrates, making the conserved interactions as described in the Results section. Superposition of the CMY-136 crystal structure on the complexes structures shows that there would be significant overlapping between the R1 sidechain of β -lactams and the H221 sidechain. This serves to explain why CMY-136 may show an increased K_m for most β -lactams: in order for the β -lactam and the enzyme to interact, either the R1 sidechain or the H221 sidechain, or both, must adopt a different conformation from the one seen in most structures of class C enzymes in complex with substrates or the CMY-136 structure, respectively. Such conformational change most likely implies an energetic cost, that in turn represents an increase in K_m . Furthermore, this higher energetic cost – and increase in K_m – for CMY-136 interacting with most β -lactams seems to correlate with how big, bulky, ramified or rigid is the R1 substituent. There are much larger increases in K_m for cloxacillin, ceftazidime, cefuroxime and cefotaxime than for those with a simpler R1 sidechain like benzylpenicillin or cefalotin. One possible explanation as for why such K_m increase is less intense or doesn't exist for CEF or FOX compared to CAZ or CXM, is that the R1 sidechain of the former is not as bulky, ramified, or rigid as for the others, and thus they may prove easier to accommodate inside the active site of CMY-136 with its Y221H mutation, implying less movement restrain for H221, or making more favourable contacts and hydrogen bonds, and thus with a lesser cost in terms of binding energy and K_m .

The analysis of MD simulations suggests that the CMY-136 structure may show increased flexibility around the R1 and R2 pockets in the active site. As previously proposed by other authors^{18,19}, an increased k_{cat} may be a consequence of an increased protein flexibility, that may allow the acyl-enzyme complexes to more often adopt a conformation competent for being attacked by the hydrolytic water and be released. In the case of CMY-136, a change in the Ω -loop conformation, widening the active site cavity by displacing V211, may also participate in the k_{cat} increase observed for certain substrates. As observed by the superposition and RMSD analysis of the MD simulations, the CMY-136 Ω -loop tends to adopt a conformation more similar to the one

observed in the CMY-136 structure than to that observed in the CMY-2 structure, regardless of the starting conformation. For CMY-2 starting with the Ω -loop in its native conformation, some fluctuation is observed but finally a conformation more similar to this native one than to the one in CMY-136 crystal structure is adopted. As mentioned earlier, this is not observed for the CMY-2 simulation starting with a CMY-136 Ω -loop conformation. In this simulation the loop remains more flexible and the Y221 sidechain adopts different conformations, as observed in the case of CMY-136. The stability of the native Y221 conformation (conserved in class C structures) in the CMY-2 MD simulation, and the fact that this conformation is not reached in a 10 ns simulation starting from a different conformation, may suggest that the native conformation of the Ω -loop of class C enzymes is quite stable, and a relatively high energetic barrier would have to be overcome to reach, or come out of it. In contrast, H221 in CMY-136 easily adopts several conformations throughout the simulations, starting from either crystal structure conformations (CMY-2 and CMY-136). This in turn may help to explain the experimental data observed, i.e. a flexible H221 would allow CMY-136 to hydrolyse β -lactams in spite of its conformation in the crystal structure. Were H221 to remain in this conformation, CMY-136 would most likely be incapable of interacting with its substrates, especially those with a more rigid, bulkier, ramified R1 sidechain. The fact that H221 can adopt different conformations during the MD simulation, one of them similar to the Y221 conformation in CMY-2, helps to imagine how it may still accommodate the substrates in its active site cavity. On the other hand, this flexibility may also explain the increase in K_m observed for most β -lactams. Although H221 seems to be much more flexible than Y221 and may adopt several conformations, once CMY-136 is interacting with the substrate, H221 would probably lose much of its movement freedom. This would imply an energetic cost, and an increase in K_m for the reaction. Such increase in K_m would clearly not be observed in the case of CMY-2 or other class C enzymes with Y221 in its native conformation, as it is probably static enough that no movement restraint is imposed on it upon binding of the substrate.

CONCLUSION

CMY-136 is a novel β -lactamase, which belongs to the CMY-2-like family. It possesses a single unusual mutation, Y221H, in a position highly conserved in all class C β -lactamases. We compared here its genetic environment to that of the closest plasmid harbouring CMY-2 and characterized CMY-136 both biochemically and structurally. Finally, *in silico* modelling techniques suggested a possible structural explanation for the differences observed compared to CMY-2. These results allowed us to better assess the potential of class C β -lactamases for their spectrum extension and for their potential threat to public health. Finally, the identification of the Y221H mutation in a natural variant of CMY-2 and which confers resistance to ceftolozane/tazobactam prior to the use of this latter antibiotic in clinics is worrisome, since it may indicate a more rapid appearance of resistance, as this type of enzyme may be selected.

EXPERIMENTAL PROCEDURES

Bacterial strains

*bla*_{CMY-136} was recovered from a urinary tract *E. coli* isolate EC13⁶. *bla*_{CMY-2} was recovered from the *E. cloacae* ec-204 strain (Bicêtre strain collection). *E. coli* TOP10 (Invitrogen, Saint-Aubin, France) and *E. coli* BL21 (DE3) (Novagen, VWR International, Fontenay-sous-Bois, France) were used for cloning experiments and protein overproduction, respectively.

Plasmid extraction and cloning

Plasmid DNA from *E. coli* EC13 was extracted using the Kieser method⁷. The Kieser extracted DNA was used to transform *E. coli* TOP10 strain by electroporation. The electroporants were plated on TSA plate containing ampicillin (100 μ g/ml). Transformants were analyzed by PCR using the primers CMY-2A and CMY-2B (5'-aaaaacatatgatgaaaaaatcggtatgctgc-3' and 5'-aaaaggatccttattgcagcttttcaagaatgc-3', respectively). From the transformants harbouring *bla*_{CMY-2-like}

genes, plasmid DNA was extracted using Kieser's method and subsequently analysed on a 0.7% agarose gel stained with ethidium bromide. Plasmids of ca. 154, 66, 48, and 7 kb of *E. coli* NCTC 50192 were used as plasmid size markers²⁰.

Plasmid sequencing (WGS)

Plasmid DNA was extracted from an *E. coli* TOP10 clone harbouring the wild type pCMY-136 plasmid using the QIAGEN Plasmid Maxi Kit following the manufacturer's instructions. The DNA concentration and purity were controlled by a Qubit® 2.0 Fluorometer using the dsDNA HS and/or BR assay kit (Life technologies, Carlsbad, CA, US). The DNA library was prepared using the Nextera XT-v3 kit (Illumina, San Diego, CA, US) according to the manufacturer's instructions and then run on Miseq (Illumina) for generating paired-end 300-bp reads. De novo assembly was performed by CLC Genomics Workbench v9.5.3 (Qiagen, Hilden, Germany) after quality trimming (Qs ≥ 20) with word size 34.

The acquired antimicrobial resistance genes were identified by uploading the assembled plasmid to the Resfinder server v2.1 (<http://cge.cbs.dtu.dk/services/ResFinder-2.1>)²¹. Plasmid incompatibility group was obtained by uploading the plasmid sequence to PlasmidFinder server 1.3 (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>)²². Plasmid sequence was blasted (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>)²³ and compared to the best hit using Artemis Comparison Tool²⁴.

Cloning of *bla*_{CMY-136} and *bla*_{CMY-2} genes

PCR amplification of *bla*_{CMY-136} and *bla*_{CMY-2} genes was performed using total DNA extraction of *E. coli* EC13 and *Enterobacter cloacae* ec-204, and primers CMY-2A (5'-aaaaacatatgatgaaaaaatcggtatgctgc-3') and CMY-2B (5'-aaaaggatccttattgcagcttttcaagaatgc-3'). Amplicons were cloned into pCR®-Blunt II-TOPO® cloning plasmid (Invitrogen, Illkirch, France) under regulation by the pLac promoter. The recombinant pTOPO-cmy-136 and pTOPO-cmy-2

plasmids were electroporated into the *E. coli* TOP10 strain and then selected using TSA-plate containing kanamycin (50 mg/L). *bla*_{CMY-136} and *bla*_{CMY-2} were amplified using primers INFcmy-2A (5'-aaggagatatacatatgatgaaaaaatcggtatgct-3') and INFcmy-2B (5'-ggtggtggtgctcgaattgcagcttttcaagaatgc-3') and cloned into pET-41b(+) expression vector (Novagen, VWR International, Fontenay-sous-Bois, France) using the NEBuilder® HiFiDNA Assembly Cloning Kit (New England BioLabs® Inc, United Kingdom), following the manufacturer's instructions. Recombinant plasmids pET41b-cmy-136 and pET41b-cmy-2 were electroporated into electrocompetent *E. coli* BL21 (DE3) and selected using TSA-plates containing kanamycin (50 mg/L).

Recombinant plasmids were extracted using the Qiagen miniprep kit and both strands of the inserts were sequenced using M13 F and M13 R primers, for the pCR®-Blunt II-TOPO® plasmid (Invitrogen, Illkirch, France), and T7 promoter and T7 terminator primers, for pET-41b(+) (Novagen, VWR International, Fontenay-sous-Bois, France), with an automated sequencer (ABI Prism 3100; Applied Biosystems, Les Ulis, France). The nucleotide sequences were analysed using software available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

Antimicrobial agents, susceptibility testing and microbiological techniques

Antimicrobial susceptibilities were determined by disk diffusion technique on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the EUCAST breakpoints, updated in 2015 (<http://www.eucast.org>). Minimal inhibitory concentration (MIC) values were determined using the Etest technique (BioMérieux, Paris, France).

β-Lactamase purification

Overnight cultures of *E. coli* BL21 (DE3) harbouring either pET41b-cmy-2 or pET41b-cmy-136 recombinant plasmids were used to inoculate 2 L of BHI broth containing 50 mg/L kanamycin.

Bacteria were cultured at 37°C until reaching an OD of 0.6 at 600 nm, and protein expression was induced overnight at 25°C with 0.2 mM IPTG. Cultures were then centrifuged at 6000 g for 15 min and the pellets resuspended with 10 mL of Buffer A (20 mM PBS, 175 mM K₂SO₄, 40 mM imidazol, pH 7.40). Bacterial cells were disrupted by sonication and protein solution was clarified by centrifugation at 10,000 g for 1 h at 4°C. The supernatant was then centrifuged at 48,000 g for 1 h at 4°C. CMY-136 or CMY-2 was purified using a NTA-Nickel pseudo-affinity chromatography column (GE Healthcare, Freiburg, Germany). Elution was performed in a gradient of 0 to 100% Buffer B (20 mM PBS, 175 mM K₂SO₄, 500 mM imidazol, pH 7.40). Purity was assessed by SDS–PAGE, and pure fractions were pooled and dialyzed against 100 mM sodium phosphate buffer (pH 7.4) 50 mM potassium sulphate and concentrated up to 6.4 mg/ml using Vivaspin® columns (GE Healthcare, Freiburg, Germany). Protein concentration was determined using Bradford Protein assay (Bio-Rad) ²⁵.

Steady-state kinetic parameters

Kinetic parameters of purified CMY-136 and CMY-2 were determined at 100mM sodium phosphate buffer (pH 7.0). The k_{cat} and K_m values were determined by analysing hydrolysis of β -lactams under initial-rate conditions with an ULTROSPEC 2000 model UV spectrophotometer (Amersham Pharmacia Biotech) using the Eadie–Hoffstee linearization of the Michaelis–Menten equation. The different β -lactams were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Protein crystallization and crystallography

Initial crystallization screenings of CMY-136 were set up using the Mosquito® HTS (TTP LabTech) in four crystallization screening suites: Classics, AmSO₄, PEGs and PEGs II (Qiagen/NeXtal). Plates were incubated at 293K in the ROCK IMAGER 1000 (Formulatrix, Inc).

The crystal was transferred to a cryo-protectant solution consisting of the mother liquor supplemented with 25% glycerol and flash-frozen in liquid nitrogen.

Diffraction data was collected at 100 K in a nitrogen cryostream on the PROXIMA2 beamline at the SOLEIL synchrotron (Saint-Aubin, France). The data were indexed and integrated with XDS²⁶ in the autoPROC toolbox²⁷. Data scaling was performed using AIMLESS²⁸ from the CCP4 suite²⁹. Data collection and refinement statistics are given in Table 3. The structure of CMY-136 was solved by the molecular replacement method with Phaser³⁰ using the structure of CMY-2 (PDB code 1ZC2) as a search model. The model was rebuilt manually in Coot³¹ and then refined using BUSTER-TNT³² with local noncrystallographic symmetry (NCS) restraints and a translation–libration–screw (TLS) description of B factors³³. The quality of the final refined model was assessed using MolProbity³⁴. Crystal structure images were generated using PyMOL³⁵.

Structure analysis, docking and molecular dynamics simulations

CMY-136 structure comparison to the previously published CMY-2 structure (PDB code 1ZC2) and analysis of docking results were performed with UCSF Chimera package³⁶. Covalent docking calculations on CMY-2 and CMY-136 were performed using the GOLD software, version 5.2 (CCDC suite)³⁷. Ligand structures were generated with 3D Structure Generator CORINA Classic (Molecular Networks GmbH, Nuremberg, Germany). Molecular dynamics simulations of CMY-2 and CMY-136 were performed with Gromacs v4.6³⁸ using the OPLS-AA force field³⁹.

Nucleotide sequences accession numbers and PDB deposition.

The amino acid sequence of *bla*_{CMY-136} gene and the nucleotide sequence of its natural plasmid pCMY-136 have been submitted to the EMBL/Genbank nucleotide sequence database under the accession numbers AVR61040.1 and MG844436.1, respectively. The crystallographic structure of CMY-136 has been deposited to the PDB, accession code 6G9T. Authors will release the atomic coordinates and experimental data upon article publication.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interests with the content of this article.

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Substrate specificity of OXA-48 modified by β 5- β 6 loop replacement

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Abstract

OXA-48 carbapenemase has rapidly spread in many countries worldwide, becoming thus a major health issue. As a consequence, several OXA-48-variants have been reported, differing by few amino acid substitutions or deletions, mostly in the β 5- β 6 loop. Whereas some OXA-48-variants with single amino acid (AA) substitutions display similar hydrolytic profiles, others with 4 AA deletions lost carbapenem-hydrolysis and gained expanded-spectrum cephalosporin hydrolysis. To provide experimental evidence for the role of the β 5- β 6 loop in substrate selectivity, the β 5- β 6 loop of OXA-48 was substituted by that of OXA-18, a clavulanic acid inhibited oxacillinase capable of hydrolyzing expanded-spectrum cephalosporins but not carbapenems. Unexpectedly, the hybrid enzyme OXA-48loop18 was able to hydrolyze not only cephalosporins, but still carbapenems as well (although with a lower k_{cat}), even though the β 5- β 6 loop was longer and its sequence quite different from that of OXA-48. The kinetic parameters of OXA-48Loop18 were in agreement with the MIC values. Crystallographic and molecular modeling results support the hypothesis that the β 5- β 6 loop of OXA-48 is probably an impediment for the binding of ceftazidime or other bulkier substrates, such as aztreonam. These unfavorable interactions would not occur with the conformation adopted by the β 5- β 6 loop grafted from OXA-18, thus explaining the extended hydrolytic profile observed for this mutant. Molecular modeling results suggest that this steric impediment in OXA-48 may be due not only to the presence of R214 or other sidechains, but also to the backbone conformation that the whole β 5- β 6 loop adopts due to its primary sequence. Finally, our results give further evidence not only of the participation of the β 5- β 6 loop in the carbapenem hydrolysis but also of its influence on the hydrolysis of larger β -lactams (e. g. ceftazidime).

- To be submitted to JBC.

Introduction

Ambler class D β -lactamases (DBLs), also known as oxacillinases, are active-serine β -lactamases like the Ambler class A and class C β -lactamase (1, 2). DBLs form a very heterogeneous family of enzymes, differing both at genetic and biochemical levels, with enzymes possessing low sequence identities and various substrate profiles going from narrow to extended-spectra of hydrolysis sometimes including carbapenems (3). The oxacillinases with carbapenem-hydrolyzing activity are, by far, the most interesting oxacillinases since they may contribute significantly to an acquired carbapenem-resistance trait in clinically-relevant bacteria. These Carbapenem-Hydrolyzing class D β -Lactamases (CHDL) may be divided in three groups: i) OXA-48 from *Enterobacteriaceae*; ii) OXA-23/-40/-58/-143 reported mostly from *Acinetobacter baumannii* and iii) OXA-198 from *P. aeruginosa* (4). None of these enzymes possess the ability to hydrolyze significantly both expanded-spectrum cephalosporins and carbapenems. The hydrolysis of carbapenems by CHDLs remains low, due to their poor catalytic efficiency towards those β -lactam molecules (5). The hydrolysis of imipenem, even slow, is faster than those of meropenem. Usually, the K_m values for imipenem are low, indicating that CHDLs have a very high affinity for that substrate. These enzymes confer reduced susceptibility to carbapenem in clinical isolates and usually require additional non-enzymatic (efflux, porin loss) to obtain high level carbapenem resistance.

β -Lactamase of OXA-48-type are the most worrisome, given their rapid spread in many countries worldwide and their

propensity to evolve by mutations leading to various phenotypic expressions (6). Although OXA-48 hydrolyzes penicillins at a high level, carbapenems at a low level, and shows (almost) no activity against expanded-spectrum cephalosporins (7). OXA-48 producers, initially described in *K. pneumoniae* isolates from Turkey in 2004, have since been extensively reported from all continents (8-10). OXA-48 represents 85% of the carbapenemases isolated in France (11) and numerous outbreaks have been described with associated high mortality rates (12). Since the first identification of OXA-48, different variants have been reported, differing by few amino acid substitutions or deletions. For a complete list of variants see the Beta-Lactamase DataBase (2) (<http://bldb.eu/BLDB.php?class=D#OXA>). In addition, analyzing metagenomic data revealed an additional unexpected variety of OXA-48-enzymes (13). Whereas some OXA-48-variants with single amino acid substitutions have similar hydrolytic activities as OXA-48, others, such as OXA-163, OXA-405 or OXA-247, have a four amino-acid deletion that results in the loss of carbapenem-hydrolysis and gain of expanded-spectrum cephalosporin hydrolysis (7, 14-16). They actually exhibit a substrate profile that is similar to that of OXA-18, an Extended-Spectrum β -lactamase of OXA-type (OXA-ESBL), identified in *P. aeruginosa*, with the exception that OXA-163 is not susceptible to clavulanic acid (17).

The structures of class D oxacillinases (OXA-1, OXA-2, OXA-10, OXA-13, OXA-23, OXA-48, OXA-245, OXA-405, among others), have been determined (2). Despite a remarkable sequence divergence between these oxacillinases, their overall fold is

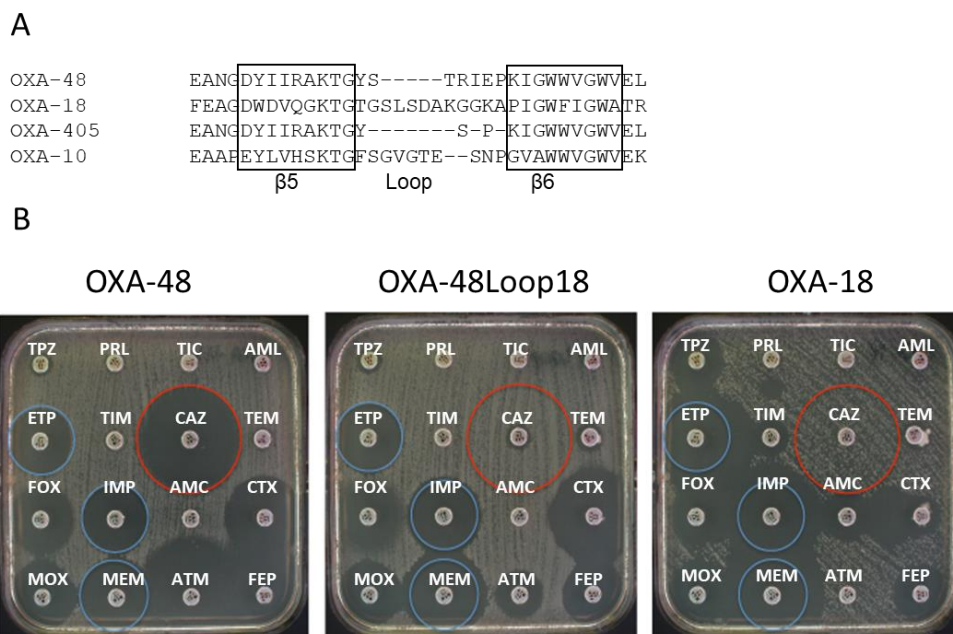


Fig 1. A) Sequence alignment of the β 5- β 6 loops of OXA-48, OXA-18, OXA-405 and OXA-10. B) Antibiograms of *E. coli* top10 harboring plasmids pTOPO-OXA-48, pTOPO-OXA-48Loop18, and pTOPO-OXA-18. TPZ: Piperacillin-tazobactam, PRL: Piperacillin, TIC: Ticarcillin, AML: amoxicillin, ETP: Ertapenem, TIM: Ticarcillin-clavulanic acid, CAZ: Ceftazidime, TEM: Temocillin, FOX: Ceftazidime, IMP: Imipenem, AMC: Amoxicillin-clavulanic acid, CTX: Cefotaxime, MOX: Latamoxef, MEM: Meropenem, ATM: Aztreonam, FEP: Cefepime. Blue circle shows imipenem hydrolysis in OXA-48, red circle shows ceftazidime hydrolysis in OXA-48.

similar and the active site elements are well conserved (18). Small differences are located mainly in the loops connecting secondary structure elements, which may vary in length and orientation (18). The orientation and size of the β 5- β 6 loop of OXA-48 is very similar to that of OXA-24/40, a CHDL from *A. baumannii*, suggesting a major role in carbapenem hydrolysis (18, 19). The replacement of the β 5- β 6 loop in OXA-10 by that of OXA-48, turned the chimeric enzyme into a carbapenemase (18, 19). This loop is close to the active site and connects two β -strands, one of which includes the catalytically relevant conserved KTG residues, which delimits one side of the active site in OXA-24 and OXA-48 (18).

To further analyse the role of the β 5- β 6 loop of OXA-48 carbapenemase in respect to the carbapenem-hydrolysis, we

substituted the β 5- β 6 loop of OXA-48 with that of the OXA-ESBL, OXA-18. The latter displays activity against expanded-spectrum cephalosporins, in unusually inhibited by clavulanic acid and is lacking significant carbapenemase activity. We evaluated the hydrolysis profile of this OXA-48Loop18 hybrid enzyme, and determined its crystallographic structure, in order to explain the observed profile.

Results

Sequence comparison of β 5- β 6 loop in relevant oxacillinases

Alignment of the β 5- β 6 loop of OXA-48 with that of OXA-10, OXA-405 and OXA-18 is shown on Fig 1A. Not only the length and the sequence of the loops are different, but also

Table 1 MIC of β -lactams for *E. coli* TOP10 pTOPO OXA-48, *E. coli* TOP10 pTOPO-48Loop18, *E. coli* TOP 10 pTOPO-18 and *E. coli* TOP10

Antibiotic	MIC (mg/l)			
	<i>E. coli</i> TOP10			<i>E. coli</i> TOP10
	(pTOPO-OXA-48)	(pTOPO-OXA-48Loop18)	(pTOPO-OXA-18)	
Amoxicillin	>256	>256	>256	2
Amoxicillin + CLA ^a	>256	32	8	2
Piperacillin	>256	>256	>256	1.5
Cefotaxime	0.75	0.19	>32	0.06
Ceftazidime	0.19	48	>256	0.12
Cefepime	0.19	2	12	0.023
Imipenem	0.75	0.38	0.25	0.25
Meropenem	0.25	0.094	0.047	0.016
Ertapenem	0.25	0.25	0.094	0.003
Temocillin	>1024	64	96	4
Aztreonam	0.047	8	>256	0.047

^aCLA, clavulanic acid (2mg/L).

the hydrolysis profile. Reducing the length of the loop of OXA-48, by 4 amino acids results in an enzyme that lost carbapenem hydrolysis, but gained expanded-spectrum cephalosporine hydrolytic activity. OXA-10 has a longer loop, and displays a broad-spectrum profile lacking any significant carbapenem and displaying low level expanded-spectrum cephalosporine hydrolysis. The loop of OXA-18, is even longer, but displays high expanded-spectrum cephalosporine hydrolysis and strong clavulanic acid inhibition, which is unusual for oxacillinases (3).

Alteration in the susceptibility profile of OXA-48.

The antimicrobial susceptibility profiles, as determined by disk diffusion (Fig 1B) and minimal inhibitory concentrations (MICs) determination for several β -lactams, conferred by OXA-48, OXA-18 and the hybrid

OXA-48Loop18 was obtained by cloning the corresponding genes in pCR-Blunt II-Topo kit (Invitrogen) and expressing them into *E. coli* TOP10 (Table1). The three proteins conferred resistance to penicillins. OXA-18 conferred a typical profile of extended-spectrum β -lactamase (ESBL), as previously described (17), meaning resistance to expanded-spectrum cephalosporins and synergy with clavulanic acid. *E. coli* Top10 (pTOPO-OXA-18) presented very low MIC values for carbapenems. On the other hand, OXA-48 conferred a reduced susceptibility to carbapenems and very low MIC values for expanded-spectrum cephalosporins (8). Interestingly, *E. coli* Top10 (pTOPO-OXA-48Loop18) presented a susceptibility profile that seems to be a combination of both. It was resistant to penicillins, ceftazidime and presented a reduced susceptibility to cefepime (MIC 2 mg/L). Additionally, a

Table 2. Steady-state kinetic parameters of β -lactamases OXA-48, OXA-48Loop18 and OXA-18.

Substrate	$K_m(\mu\text{M})$			$k_{cat}(s^{-1})$			$k_{cat}/K_m (mM^{-1}/s^{-1})$		
	OXA-48 ^a	OXA-48Loop18	OXA-18	OXA-48 ^a	OXA-48Loop18	OXA-18	OXA-48 ^a	OXA-48Loop18	OXA-18
Ampicillin	395	43	8	955	4	2.1	2418	92	259
Cefalotin	195	124	42	44	0.5	34	226	4.3	804
Cefoxitin	>200	77	9	>0.05	0.004	0.302	0.3	0.05	35
Ceftazidime	N.H	>1000	44	N.H	>0.2	10	N.H	0.2	236
Cefotaxime	>900	>1000	54	>9	>3.1	62	10	2.4	1158
Cefepime	>550	357	78	>0.60	0.4	19	1.1	1.1	248
Imipenem	13	109	13	5	0.4	0.006	369	3.2	0.5
Meropenem	11	16	71	0.07	0.01	0.01	6	0.7	0.2
Ertapenem	100	15	12	0.13	0.02	0.01	1.3	1.1	0.9
Aztreonam	N.H	>1000	14	N.H	>1.3	3.3	N.H	0.7	234

NH, Hydrolysis could not be detected with concentrations of substrate and enzymes up to 1000 mM and 400 nM, respectively.

^a values from Docquier *et al* (18)

synergy with clavulanic acid was also observed but not as marked as with *E. coli* Top10 (p-TOPO-OXA-18). At the same time, it showed lower MIC values for carbapenems as compared to those of *E. coli* Top10 (pTOPO-OXA-48) but higher to those of *E. coli* Top10 (pTOPO-OXA-18). In a similar manner, MIC values for temocillin and aztreonam ranged between the native enzymes.

Kinetic analysis

To evaluate the biochemical properties, steady-state kinetic parameters were determined to compare the catalytic efficiencies of OXA-48 with that of OXA-18 and OXA-48Loop18 (Table 2). OXA-48 presented the highest catalytic efficiency for ampicillin, followed by that of OXA-18 and OXA-48Loop18. In the last two cases, the reduced catalytic efficiencies were due to lower k_{cat} even though the K_m s were at least 10-fold lower. Unlike OXA-48, OXA-48Loop18 was able to hydrolyze ceftazidime, with a lower catalytic efficiency than OXA-18. These kinetic data are in agreement with the observed MIC values. For imipenem, the highest catalytic efficiency was observed

with OXA-48, whereas that of OXA-48Loop18 was 100-fold lower, as a result of a 10-fold higher K_m and a 10-fold higher k_{cat} . Interestingly with meropenem, and even more with ertapenem, these hydrolytic differences between OXA-48 and OXA-48Loop18 were less important. For Ertapenem, similar values were found as a consequence of 10-fold lower K_m for OXA-48-Loop-18, even though the k_{cat} was 6-fold lower. These small differences were enough to modify the MIC values from 0.094 mg/L (conferred by OXA-18) to 0.25 mg/L (conferred by OXA-48Loop18 and OXA-48).

OXA-48Loop18 crystallization and X-ray crystallography.

OXA-48Loop18 crystallized with a buffer solution containing 2.2 M ammonium sulfate and 0.2M ammonium fluoride. The structure was determined by molecular replacement using the deposited OXA-48 structure (PDB: 4S2K) as a template, and the model was refined to 2.38 Å (Table 3). The asymmetric unit contains two protein chains, A and B, modelled with 252 residues each. Both chains present the typical class D fold with an α -helical region and a mixed

Table 3. Crystallography data collection and refinement statistics.

Data collection	
Space group	P 2 ₁ 3
Cell dimensions	
a, b, c (Å)	126.65, 126.65, 126.65
α, β, γ (°)	90.00, 90.00, 90.00
Resolution (Å)	19.78-2.38
R _{meas}	14.6%
I/σ(I)	1.41 (at 2.38Å)
Completeness (%)	99.3
Redundancy	16.3
Refinement	
Resolution range (Å)	19.78-2.38
No. unique reflections	27,144
R _{work} /R _{free}	17.1%/21.4%
No. non-hydrogen atoms	
Protein	4,088
Water	103
Ligand/Ions	100
Total	4,291
Average B, all atoms (Å ²)	61.14
Protein	61.02
Water	56.44
Ligand/Ions	70.93
Root mean squared deviations	
Bond lengths (Å)	0.01
Bond angles (°)	1.11

α-helix/β-sheet region. 96% of all residues are inside the favored regions of the Ramachandran plot, and 4% in the allowed regions. Clear electron density is observed for most regions of the protein for both backbone and sidechains (Fig. 2A), with the exception of loops β5-β6 on chain A and β7-α10 on both chains. Weak electron density allows for a partially adopted conformation to be modelled for the backbone of these loops, as well as the general orientation of the sidechains.

Several ordered water molecules have been modelled in the structure as well as sulfate and fluoride from the crystallization solution, and glycerol from the cryoprotectant. No significant differences can be observed between both chains, except for the conformation adopted by the flexible loops. In chain B, the β5-β6 loop adopts an elongated conformation, extending away from the protein (Fig. 2B). In chain A, the same loop folds back towards the Ω-loop. β7-α10 loop in chain B seems to partially adopt a conformation similar to that of OXA-48 structures, although slightly shifted, possibly because of the β5-β6 loop replacement. In chain A, the same loop seems to adopt a conformation where the N-terminal region of helix α10 is unwound and the loop seems to close the active site cavity. This suggests that these two loops, β5-β6 and β7-α10, may be quite flexible in OXA-48Loop18, whereas for OXA-48 both loops seem to be well defined and in the same conformation in almost all deposited structures. The active site serine of both chains, S70, has been partially modelled as O-Sulfo-L-serine (OSE), with an occupancy of 0.5 (Fig. 2A). To the best of our knowledge, this is the first example of a β-lactamase structure with a partially sulfonylated S70. A few other cases of structures with OSE have been deposited in the PDB (accession codes 5V8D, 1EA7, 1YLN, 4HF7). In the publications available for these structures, the authors have proposed that the OSE may arise as an in-situ modification inside the crystal and its presence does not alter the conformation of the rest of the protein. Except for PDB 1EA7, for which HEPES buffer was suggested responsible for the serine modification (20), the three other structures were obtained using high sulfate concentrations as crystallizing reagent. Inside the PatB1 crystal (PDB core5V8D), the authors propose that an equilibrium between OSE and a sulfate ion buried in the active site next to the unmodified serine (21). Similar to our β-lactamase

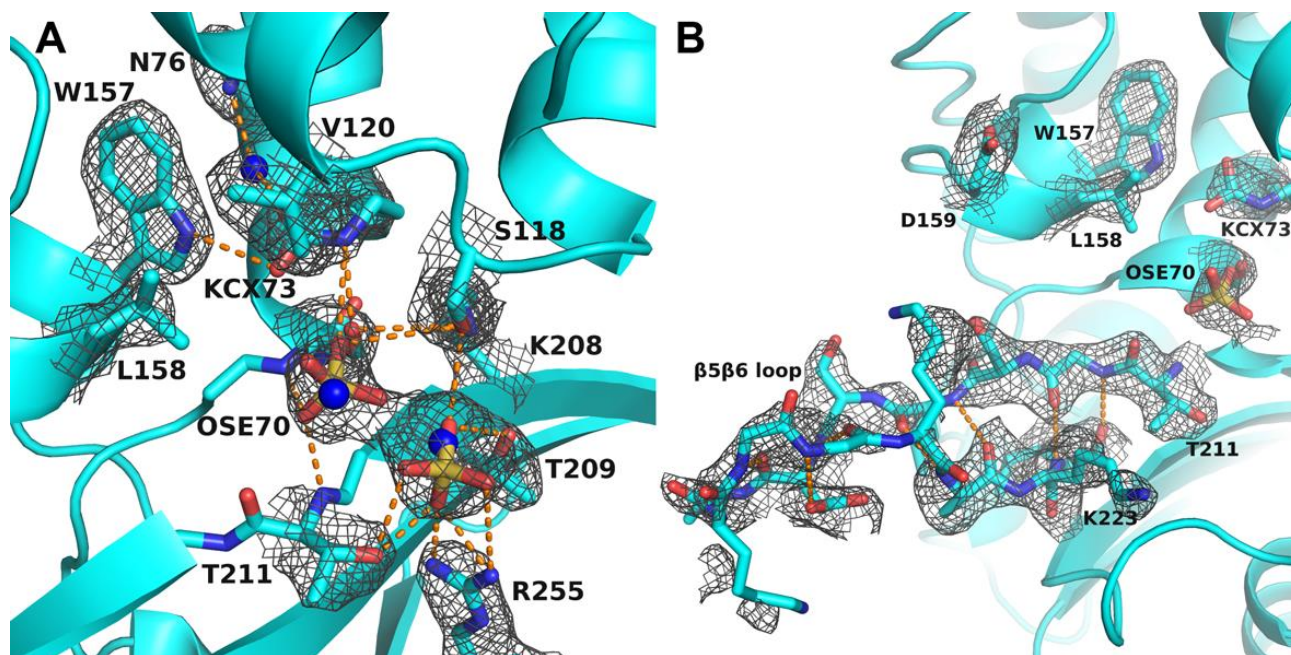


Fig. 2. Crystal structure of OXA-48loop18. A) Clear electron density can be observed for residues in the active site cavity. Water molecules are represented in blue to evidence their superposition with the sulfate molecules. Hydrogen bonds are depicted as orange dashed lines. B) Clear electron density is observed for the backbone of the $\beta 5$ - $\beta 6$ loop as well as for most sidechains. Hydrogen bonds, depicted in orange, show the extension of the β -sheet.

structure, they have modelled OSE at 0.5 occupancy, with one of the oxygen atoms buried inside an oxanion hole, arguing that this sulfonylation supports the proposed role of catalytic nucleophile for the serine in PatB1. In the OXA-48Loop18 structure, another sulfate can be observed in the active site, modelled at 0.5 occupancy, making hydrogen bonds to R255 (R250 in OXA-48), T209, T211 (Y211 in OXA-48) and S118. Only the sulfate anion or the OSE is proposed to occupy the active site cavity at any given protein monomer in the crystal, and a water molecule, also modelled at 0.5 occupancy, would occupy the place of the other.

OXA-48Loop18 shows the same overall conformation as OXA-48 (PDB 4S2P), and 3D alignment reveals a C α RMSD of 0.464 Å between them, excluding the exchanged $\beta 5$ -

$\beta 6$ loop. The sidechains of the active site cavity residues adopt the same conformation as in the OXA-48 structure (Fig. 2A). A few conformational differences can be found when comparing both structures: (i) the $\beta 5$ - $\beta 6$ loop of OXA-48Loop18, grafted from the OXA-18 sequence, is five residues longer than the native loop of OXA-48, and adopts a more relaxed and elongated conformation (Fig. 2B). Superposition of residues 212 to 214 of OXA-48 $\beta 5$ - $\beta 6$ loop on OXA-48Loop18 $\beta 5$ - $\beta 6$ loop shows that only minor clashes with the omega loop would occur, between R214 and D159 sidechains. Attempts to build a model of OXA-48 on either chain of OXA-48Loop18 using Modeller (22) show that an elongated conformation for the native $\beta 5$ - $\beta 6$ loop of OXA-48 would require a trans E216-P217 bond, with outlier Ramachandran values, as well as slightly separating the $\beta 5$

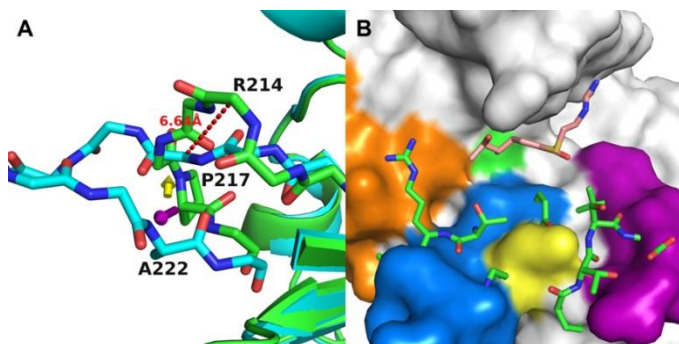


Fig. 3. Structure conformational differences of OXA-48loop18 and OXA-48. A) Differences in conformation of the $\beta 5$ - $\beta 6$ loop of OXA-48 and OXA-48loop18. A 6.64 Å shift in the $C\alpha$ of residue 214 is observed. OXA-48 is colored in green and OXA-48loop18 in cyan. Yellow and purple arrows point towards the 216-217 peptide bond of OXA-48, in cis configuration, and OXA-48loop18, in trans configuration, respectively. B) Surface representation of OXA-48loop18 superposed on OXA-48 (green sticks), with imipenem (coral sticks) docked in the active site for reference. Surface is colored according to structural features (orange: Ω -loop; blue: $\beta 5$ - $\beta 6$ loop; yellow: S223; purple: $\beta 7\alpha 10$ loop; green: S70). Notice how the OXA-48 structure emerges from the OXA-48loop18 surface, exhibiting a narrower active site cavity.

and $\beta 6$ strands, and breaking the last 3 hydrogen bonds formed between them. It would also force R214 sidechain to be directed in the opposite direction it usually does, losing its interaction with D159. Such large conformational change would most likely not occur. This supports the idea that the conformation adopted by the $\beta 5$ - $\beta 6$ loop of OXA-48 may be a consequence of its short length, the constrain imposed by P217, and the interaction of R214 and D159 (18). The $\beta 5$ - $\beta 6$ loop of OXA-18 lacks P217 and R214 and is five residues longer. As a result, the loop may adopt a more flexible and elongated conformation with a shift of 0.56 Å, 0.75 Å, 3.12 Å and 6.64 Å in the $C\alpha$ of residues 211 through 214, respectively (Fig. 3A), extending the $\beta 5$ and $\beta 6$ strands and protruding away from the active site cavity. In turn, this means that the R214 barrier delimitating the active site cavity of OXA-48 is lost in OXA-48Loop18. The loop exchange also allows the backbone of residues T211, G212, and S213 (YST in OXA-48) to shift slightly towards the Ω -loop and closer to the $\beta 6$ strand, widening the active site cavity due to the shift

of backbone and sidechain positions. The exchange of Y211 for T211 also leaves a shallow cleft between the $\beta 5$ strand, at the bottom of the active site cavity, and the wall formed by the $\beta 7$ - $\alpha 10$ loop, which may be partially occupied by K223 (K218 in OXA-48) (Fig. 3B).

Covalent docking of β -lactams on OXA-48Loop18 and comparison with OXA-48.

Covalent docking of the open β -lactams' acylating structure was performed, for both OXA-48Loop18 and OXA-48, to explore what conformation the acyl-enzyme complexes could adopt after initial hydrolysis that may explain the acquired capacity of OXA-48Loop18 for ceftazidime hydrolysis, and the difference in K_m for imipenem. Covalent docking conformations on OXA-48 for ceftazidime show unrealistic results for all solutions offered by GOLD (23), with the β -lactams orientation inverted (R2 sidechain towards the Ω -loop and R1 sidechain towards K208) or the R1 aminothiazole ring lodged inside a narrow cavity between the $\beta 5$ strand and the Ω -loop, showing severe clashes. These results were expected, as the OXA-48 cavity is supposed to be too small to bind ceftazidime. The OXA-48Loop18 structure, in contrast, shows docking results with conserved interactions (R1 and R2 sidechains positioned towards the corresponding pockets, R1 amide making a hydrogen bond with Thr211 O, β -lactam carbonyl oxygen buried in the oxyanion hole, C3/C4 carboxylate binding R250), and resembling conformations adopted for OXA-160 (PDB code 4X56) and, especially, OXA-225 (PDB code 4X55) (24) (Fig. 4A). Superposition of the OXA-48 structure on the docking results on OXA-48Loop18 illustrates

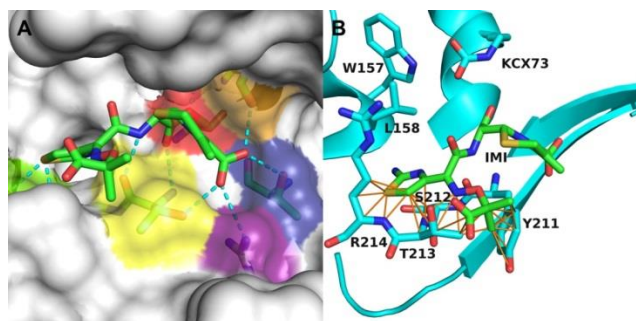


Fig. 4. Docking of ceftazidime on OXA-48loop18. A) Docking conformation of ceftazidime on OXA-48loop18, making hydrogen bonds to S70 (in red), S118 (in orange), T209 (in blue), R255 (in purple), T211 (in yellow), and S213 (in green). The docking conformation is similar to the one observed for the complex of OXA-225 with ceftazidime (PDB code 4X55). B) Superposition of OXA-48 on OXA-48loop18 with ceftazidime docked, showing overlaps between the R1 sidechain of ceftazidime and residues 211-214 of OXA-48. Overlaps are represented as thin orange lines and range from 2.0 to 0.6 Å.

how the conformation of the $\beta 5$ - $\beta 6$ loop backbone and sidechains represents a clear steric impediment for the binding of the ceftazidime in the active site (Fig. 4B). There would be severe clashes between the R1 substituent of ceftazidime and Y211, S212, T213, and R214, with distances between overlapping atoms ranging from 2.0 to 0.6 Å. These results would explain the acquired capacity of OXA-48Loop18 of hydrolyzing β -lactams with bulky R1 sidechains like ceftazidime and aztreonam, which are not hydrolyzed by OXA-48, due to the conformation adopted by the $\beta 5$ strand and $\beta 5$ - $\beta 6$ loop. These results suggest that the conformational changes in these positions, product of the loop exchange, may be responsible for the expansion of the hydrolytic profile.

Superposition of OXA-48Loop18 on docking results of imipenem with OXA-48 or the crystallized structure of OXA-48 with imipenem (PDB 5QB4) show minor clashes between the R1 substituent and OXA-48Loop18 sidechains S118, V120, and L158. This may in part be responsible for the increased K_m of OXA-

48Loop18 for imipenem, together with the widening and increased flexibility of the active site cavity, which may not necessarily decrease K_m for a small substrate like imipenem, in contrast to the effect on ceftazidime or other bigger substrates.

Molecular dynamics simulations of OXA-48 and OXA-48Loop18

Molecular dynamics (MD) simulations on the apo form of both enzymes were performed, showing that the OXA-48Loop18 $\beta 5$ - $\beta 6$ loop is more flexible than that of OXA-48, and may alternate between the conformations observed on both chains of the crystal structure. RMSF analysis of the simulation reveals differences in the flexibility of several parts of the protein (Fig. 5A and 5B). The $\beta 7$ - $\alpha 10$ loop seems to be more flexible than in OXA-48 as well. This is in agreement with the only partially occupied conformation for the $\beta 7$ - $\alpha 10$ loops observed in the crystal structure. K223 also shows increased flexibility compared to K218 in OXA-48. This may be due to the fact that in the place of E216 the OXA-48Loop18 has K221, and both sidechains may repel each other. Movement of K223 towards the active site cavity may allow it to form a salt bridge with the R1 carboxylate of ceftazidime, improving its affinity. This increased flexibility of K223, and the whole $\beta 5$ - $\beta 6$ loop, may be directly responsible for the flexibility of the $\beta 7$ - $\alpha 10$ loop. Increased flexibility is also observed for S118 and L158. Both loops on the other side of the cavity (comprising S118 and V120, and I102 and W105) show increased flexibility as well, compared to OXA-48.

MD simulations of the covalent complexes with imipenem were performed for both OXA-48 and OXA-48Loop18, to explore the

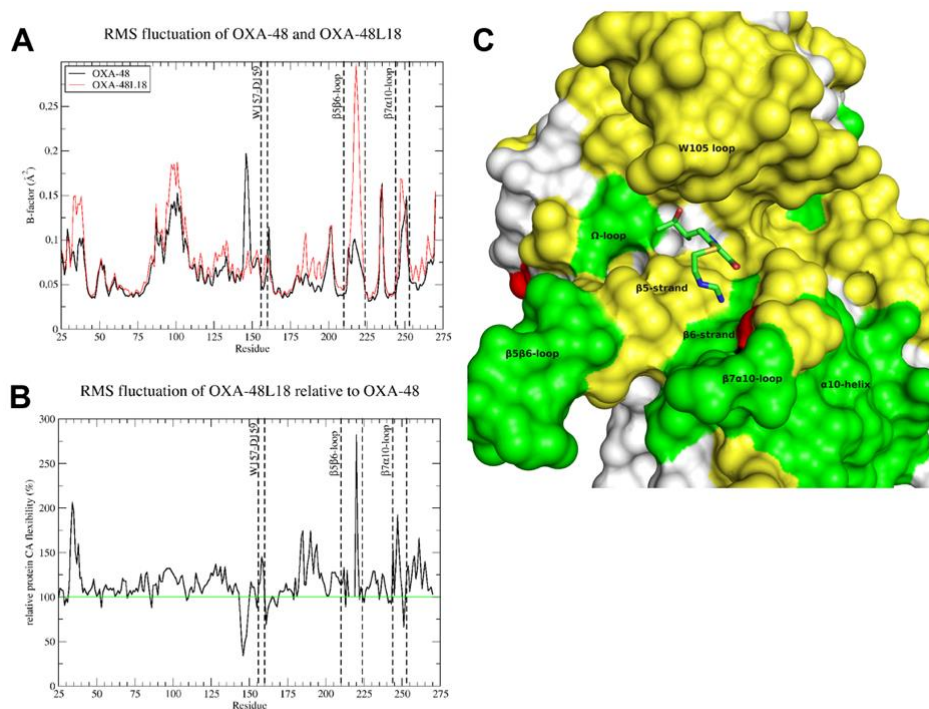


Fig. 5. Flexibility of OXA-48loop18. A) RMSF analysis of the OXA-48 and OXA-48loop18 simulations, expressed as C α B-factors. OXA-48loop18 shows an increase in flexibility over most of the protein sequence. Notice the increase on the β 5- β 6 and β 7 α 10 loops. B) Relative flexibility of OXA-48loop18 compared to OXA-48. Notice the almost 50% increase in L158 flexibility, which may go unnoticed by looking at the absolute B-factor graph. C) Surface representation of OXA-48loop18 with imipenem. Increased flexibility has been depicted in yellow (moderate increase) and green (higher increase). Certain parts of the enzyme show decreased flexibility, such as the back of the Ω -loop and G251 in the β 7 α 10 loop.

effect the loop graft may have on the enzymatic kinetics for this substrate, which showed a 10-fold k_{cat} drop. Regarding flexibility, these simulations with the covalent complexes showed similar results with the apo simulations: several parts of the enzyme showed increased flexibility even when covalently bound to imipenem, namely the S118-V120 loop, R100-A103 loop, the W157-L158-D159 portion of the Ω -loop, the whole β 5 strand, the β 5- β 6 and β 7- α 10 loops, as well as other regions around the protein surface. The largest increases can be

observed for L158, L214-K221, and N243-L254 (Fig. 5C).

To explore how the turnover rate of imipenem may be affected in OXA-48Loop18, water molecule dynamics and the occupancy of conserved water positions during the simulation was examined with the HOP package (25). Results show the water network around the protein surface and in the active site cavity (Fig. 6). The amount of consensus sites found for apo OXA-48 (186 sites) is quite larger than for apo OXA-48loop18 (52 sites), which may be related to

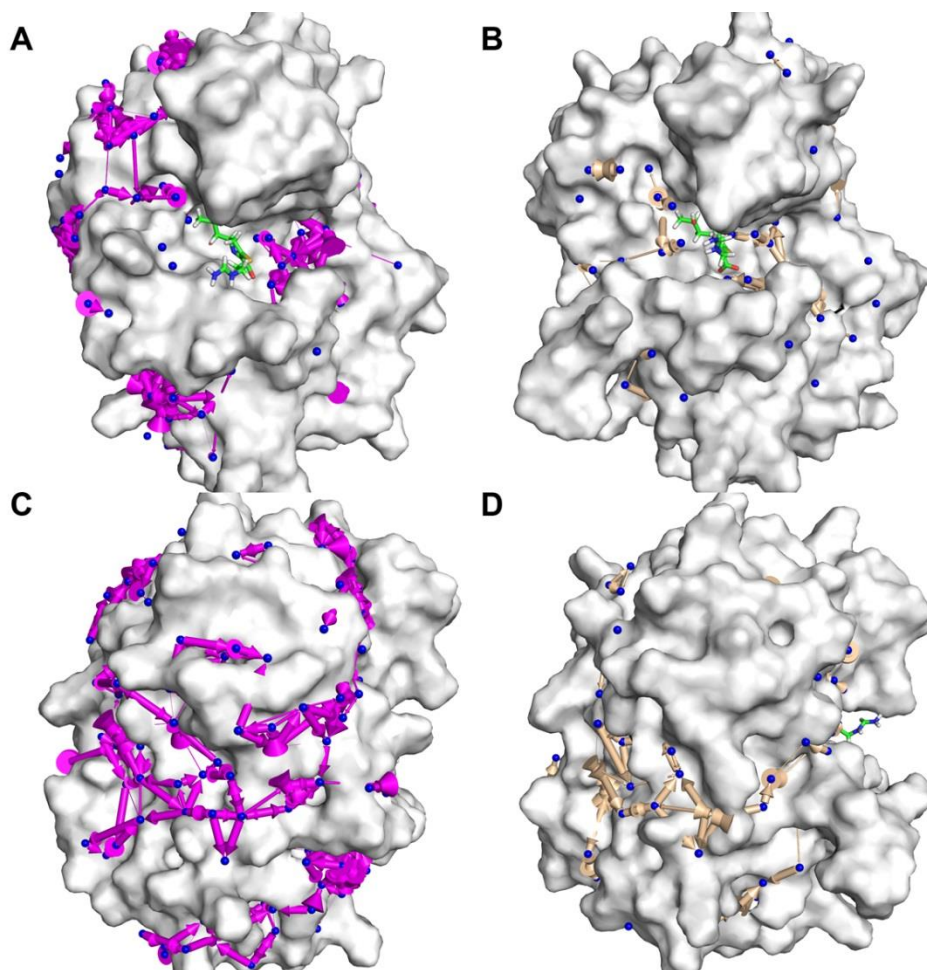


Fig. 6. Water networks around OXA-48 and OXA-48loop18. Dynamic water networks around the protein surface, determined by HOP analysis. Notice the larger amount of conserved water sites obtained for OXA-48/imipenem simulations relative to OXA-48loop18/imipenem. A) OXA-48/imipenem complex, front view. B) OXA-48loop18/imipenem complex, front view. C) OXA-48/imipenem complex, back view. D) OXA-48loop18/imipenem complex, back view.

the increased flexibility of OXA-48loop18. However, a smaller difference is observed for the imipenem complex simulations, where the sites determined amount to 191 and 126, for OXA-48 and OXA-48loop18, respectively. Visual inspection of the water network, however, suggests that the hops between sites are more discrete for OXA-48loop18 than for OXA-48, whether in the apo form (data not shown) or in complex (Fig. 6). Water positions not connected to other sites by arrows representing hop constants,

interact only with the bulk solvent. The HOP analysis also reveals conserved water positions in the active site cavity of OXA-48, and how these positions are displaced upon binding of imipenem (Fig. 7), which supports the hypothesis that the R1 group of carbapenems may push the attacking water away from an optimal attacking position. For OXA-48 a clear path for accession of a water molecule to the hydrolytic water position can be evidenced by the HOP analysis (Fig. 8A). Water molecules accessing this cavity can

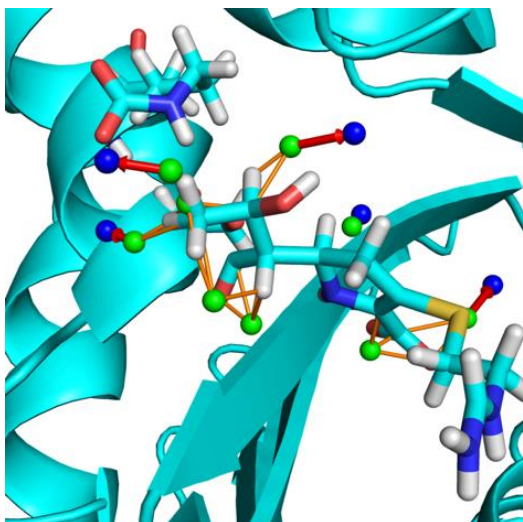


Fig. 7. Displacement of active site water molecules by imipenem. Conserved water molecule sites determined by HOP for OXA-48 (green spheres) and OXA-48-imipenem (blue spheres) simulations in complex with imipenem. Overlaps displayed with orange lines. Notice the shift of certain water molecule sites caused by the presence of imipenem in the active site (red arrows). Other water molecules are simply displaced.

originate from the bulk solvent or conserved positions around the Ω -loop surface, and are more likely to enter through a channel between L158 and Q124, by hydrogen bonding the following residues: R214 sidechain, S155 sidechain or F156 backbone, Q124 sidechain, W157 sidechain, and finally KCX73, about 2 Å above the hydrolytic water pocket. The final access to this pocket seems to be only partially allowed by the movement of the L158 sidechain and the imipenem R1 sidechain. These movements may be directly influenced by the loop exchanged, as it has been proposed that a hydrophobic patch between the β 5- β 6 loop and the Ω -loop may interact with the R1 sidechain to help it turn (18), and a similar interaction was proposed for L166 in the case of OXA-23/meropenem complex (26). In

OXA-48loop18, the β 5 strand comes closer to the Ω -loop, leaving less space in-between. During the MD simulation of OXA-48 it was also observed that S118 could turn and make a hydrogen bond with the R1 sidechain, which is possibly participating in this turning event. Once inside the pocket, the distance and angle of the active water molecule to the scissile bond is influenced by the conformation of the R1 sidechain as well. The distance is larger when the methyl group is facing towards it, and it may approach closer to the scissile bond when both the methyl and hydroxyl ends of R1 are rotated 120° away from it, or also when the R1 hydroxyl group is positioned on the β face of the β -lactam and may form a hydrogen bond with the water molecule. The consequences of the loop exchange on the water molecule distance to the β -lactam may also be affected by other surrounding residues, such as L158 which becomes fixed in a certain rotamer to allow space for the water molecule, and only can turn after it leaves, or A69 and its methyl orientation. For the OXA-48Loop18 simulation, the access to the active site seems to be through a different path (Fig. 8B). The R214 sidechain at the edge of the entering channel is not present in this β 5- β 6 loop, and the backbone of the Ω -loop is more flexible. Water molecules reaching the hydrolytic water pocket seem to originate from the bulk solvent close to the β face of imipenem, hopping through two consensus positions next to the R1 sidechain, later binding KCX73 and then accessing the pocket below. As similar Hop constants are obtained in this simulation, the flow towards the hydrolytic water pocket alone may not explain the differences in turnover rate for imipenem in this case. However, inside the pocket the average position adopted by the

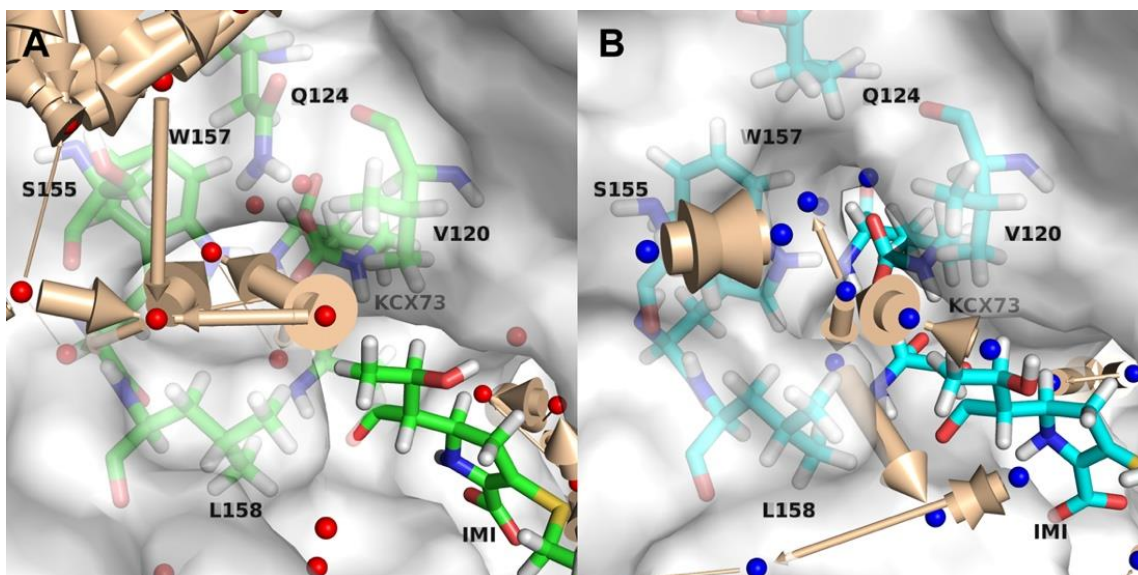


Fig. 8. Water molecules flow into the active site cavity. A) In OXA-48/imipenem complex, the access to the hydrolytic water pocket in OXA-48 seems to be through a channel above L158, hopping on 4 to 5 conserved sites. B) In OXA-48Loop18/imipenem complex, the access to the hydrolytic water pocket seems to be more direct, hopping on 3 or 4 conserved sites, arriving from the β face of the substrate. Arrow widths are proportional to water molecule hopping rates between conserved sites.

water molecules, and the average conformation of the covalently bound imipenem, are different for both covalent complexes (Fig. 9). In the case of OXA-48, the water molecule average position has an occupancy of 0.60, and is situated 3.67 Å away from the β -lactam carbon, at an angle of 70.8° from the bond plane. In the case of OXA-48Loop18, the same pocket contains a water molecule average position with an occupancy of 0.52, but it is positioned 4.54 Å away from the β -lactam carbon, and at an angle of 59.0°.

Discussion

The β 5- β 6 loop appears to have a profound influence on the hydrolytic profile of class D β -lactamases (18, 19). Point mutations on this loop and loop exchange

experiments have already been described to alter the hydrolytic profile, being able to turn non-carbapenemases into carbapenemases, as well as the opposite (7, 19). The mechanisms by which β -lactamases achieve this can depend on the enzyme characteristics and the substrates in question. The capacity to hydrolyze bulkier substrates can be acquired by turning the enzyme more flexible, usually at the cost of a decreased k_{cat} and thermal stability for the mutant (27). Substitutions of residues that may pose a steric impediment for the binding of substrates for smaller or more flexible residues can also occur, as well as mutations that may allow favorable new interactions with a certain substrate to be made (28). The β 5- β 6 loop of OXA-48 has already been proposed to be an impediment for the binding of big substrates such as

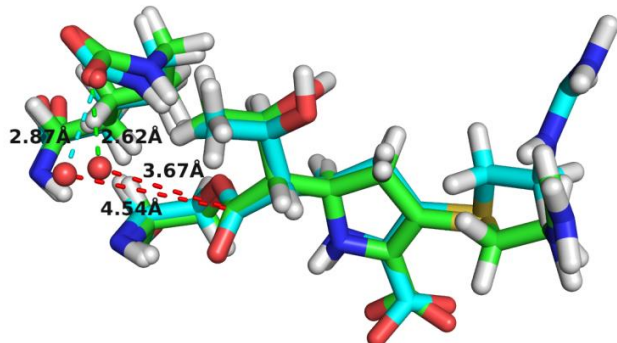


Fig. 9. Conserved water site in the hydrolytic water pocket. Conserved water position as determined by HOP analysis for OXA-48/imipenem complex (green) and OXA-48loop18/imipenem complex (cyan). The conserved position for the OXA-48loop18 enzyme is farther from both the activating KCX and the scissile bond, and at a less favorable angle (59.0° instead of 70.8°).

cephalosporins, as well as intervening in the turnover rate of carbapenems by interacting with the R1 sidechain to facilitate rotation and allow the water molecule to perform deacylation (18).

In this work, we successfully transferred the $\beta 5$ – $\beta 6$ loop of an expanded-spectrum cephalosporinase, OXA-18, to the carbapenemase OXA-48. Unexpectedly, the hybrid enzyme OXA-48loop18 was able to hydrolyze not only cephalosporins, but still carbapenems as well (although with a lower k_{cat}), even though the $\beta 5$ – $\beta 6$ loop was longer and its sequence quite different from that of OXA-48. These results give further evidence not only of the participation of the $\beta 5$ – $\beta 6$ loop in the carbapenem hydrolysis (18, 19) but also of its influence on the hydrolysis of bulkier β -lactams (e. g. ceftazidime).

We also present here crystallographic and molecular modeling results that support the hypothesis that the $\beta 5$ – $\beta 6$ loop of OXA-48 is probably an impediment for the binding of ceftazidime or other bulky substrates, such as aztreonam. These unfavorable interactions would not occur with the conformation adopted by the $\beta 5$ – $\beta 6$ loop grafted from OXA-18, thus explaining the extended hydrolytic profile observed for this mutant. Molecular modeling results suggest that this steric impediment in OXA-48 may be due not only to the presence of R214 or other sidechains, but also to the backbone conformation that the whole $\beta 5$ – $\beta 6$ loop adopts due to its primary sequence. The flexibility that the loop exchange seems to cause on other parts of the protein may play a part in the hydrolytic profile expansion as well. The increased flexibility of L158, for example, may also collaborate in admitting larger substrates into the active site cavity of OXA-48Loop18. The K_m of imipenem is also affected by the loop exchange. Covalent docking calculations show slight clashes between the imipenem docking pose on OXA-48 (which is similar to the imipenem conformation in the PDB structure 5QB4), and the L158, V120 and S118 sidechains of OXA-48Loop18. The docking pose of imipenem on OXA-48Loop18 is also slightly shifted, possibly to avoid producing these overlaps. These small clashes however, may not be enough to explain a 10-fold increase in K_m . However, unlike for ceftazidime, the widening and increased flexibility of the active site cavity may not have a positive impact on a small substrate such as imipenem, for which the OXA-48 structure was already well adapted. The R1 substituent of carbapenems, for example, is already

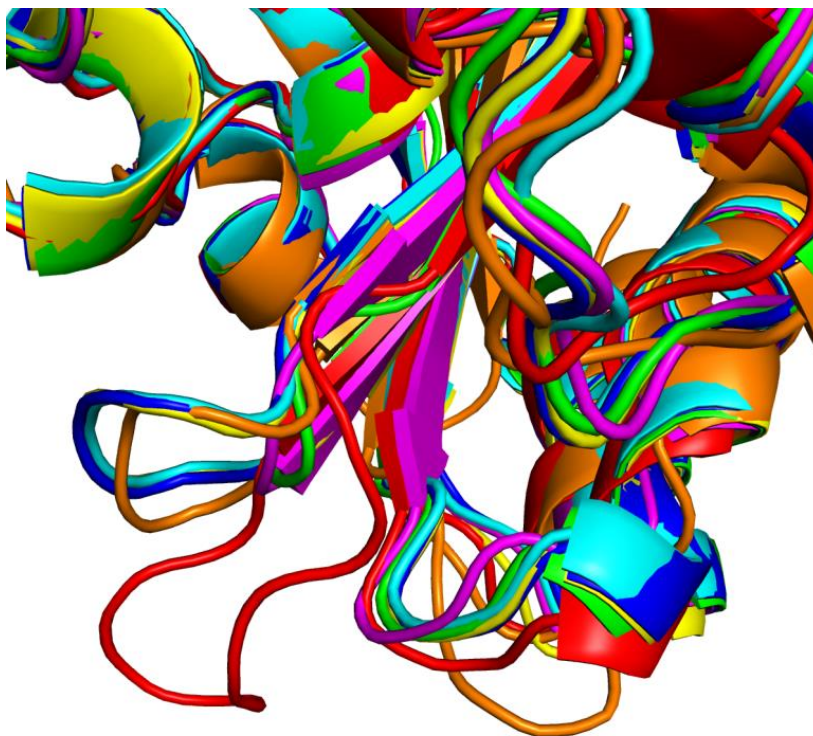


Fig. 10. $\beta 5$ - $\beta 6$ loop conformation of OXAs with different hydrolytic profiles. $\beta 5$ - $\beta 6$ loop conformation adopted by different OXA type enzymes. Red: OXA-10, green: OXA-163, blue: OXA-181, yellow: OXA-245, magenta: OXA-405, cyan: OXA-48, orange: OXA-51. Notice the almost identical conformation of the OXA-181, OXA-245 and OXA-48 loops.

known to interact directly with V120 and L158, and our simulations show that it also can transiently turn and make a hydrogen bond with S118. Such interaction may also participate in helping deacylation, by promoting the rotation of the R1 sidechain. Moreover, considering that the entire $\beta 5$ - $\beta 6$ loop of OXA-48 was replaced for a 5 AA longer one with a different amino acid composition and that imipenem hydrolysis was still observed, our results suggest that the presence of the exact $\beta 5$ - $\beta 6$ loop of OXA-48 or similar is not absolutely fundamental for the hydrolysis of carbapenems. Its three-dimensional

conformation however, may be. It was shown that OXA-48 and OXA-24, both CHDL, present the same conformation of the $\beta 5$ - $\beta 6$ loop (18). The same situation is observed in the crystal structure of OXA-181 (29) and OXA-245 (29). At the same time, the superposition of OXAs with expanded spectrum cephalosporinase activity and non-carbapenemase activity shows heterogeneity in the disposition of the $\beta 5$ - $\beta 6$ loop (Fig. 10). This might suggest, firstly, that only the OXAs with the $\beta 5$ - $\beta 6$ loop in the OXA-48 loop conformation, or close to it, would be able to hydrolyze carbapenems, while OXAs presenting different loop conformations

would hydrolyze expanded spectrum cephalosporins. Secondly, there are loop sequences that produce intermediate states (expanded hydrolysis spectrum), such as for our hybrid protein, that can hydrolyze both types of substrates (although with a lower k_{cat} for carbapenems), with a $\beta 5$ – $\beta 6$ loop quite different in primary sequence from the one of OXA-48, but more similar in orientation to it than the $\beta 5$ – $\beta 6$ loop of other OXAs, such as OXA-10. Lastly, if only the modification of the $\beta 5$ – $\beta 6$ loop of OXA-48 is sufficient for it to start hydrolyzing expanded-spectrum cephalosporins, this suggests that the rest of the enzyme active site residues present a pertinent configuration and conformation for the acylation and deacylation process of cephalosporins. Thus, our results provide further evidence that the $\beta 5$ – $\beta 6$ loop of OXA-48 would influence the hydrolytic profile in two ways: (i) presenting a steric impediment for cephalosporins or other bulky substrates, and (ii) promoting the deacylation step of carbapenems, possibly by interacting with the R1 sidechain so as to facilitate the hydrolytic water attack (18). In this case, the loop exchange seems to have an effect on k_{cat} , for imipenem at least, by altering the attacking water occupancy within the pocket, and its position relative to the substrate. The HOP analysis shows that the route a water molecule is most likely to follow to enter the active site cavity on both enzymes is different, but with similar water hopping rates. The difference in k_{cat} is explained by the different position of the active water compared with the acyl moiety. The exact relation between the $\beta 5$ – $\beta 6$ loop sequence and the water molecule competence for deacylation should be further explored. It is possible that the same increased flexibility of

the $\beta 5$ – $\beta 6$ loop, or other parts of the enzyme, that allow for a hydrolytic profile expansion may also have an effect on the turnover rate for all substrates, by affecting the catalytic mechanism at a certain step for all of them. The position, flexibility, or difference in residues, of the grafted loop may interact differently than the loop of OXA-48 with imipenem. The increased flexibility of L158 in OXA-48loop18, also a consequence of the loop exchange, may also generally affect the turnover rates of all substrates by increasing competition for space within the hydrolytic water pocket. Indeed, visual inspection of the MD simulation indicates that L158 is restricted from rotating while the water molecule is positioned inside the pocket. Further analysis needs to be performed to precisely determine how the loop sequence affects this, as well as other possible molecular mechanism through which the loop sequence may affect the turnover rate or affinity for other substrates.

Experimental procedure

Bacterial strains. *K. pneumoniae* 11978 was used as a reference strain for OXA-48 cloning experiments (8). *P. aeruginosa* Mus was used as a reference strain for OXA-18 cloning experiments (17).

Antimicrobial agents, susceptibility testing and microbiological techniques. Antimicrobial susceptibilities were determined by disk diffusion technique on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the EUCAST breakpoints, updated in 2018 (<http://www.eucast.org>). Minimal inhibitory concentration (MIC) values were determined using the Etest technique (BioMérieux, Paris, France).

PCR, cloning experiments, and DNA sequencing. Whole-cell DNAs of *K. pneumoniae* 11978 isolate and of *P. aeruginosa* Mus were extracted using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France) and were used as template for PCR using

the following primers: preOXA-48A (5'-TATATTGCATTAAGCAAGGG-3'), cloningOXA-48B (5'-AAAAGGATCCCTAGGGAATAATTTTTCTGTTGA GCA-3') and preOXA-18Fw (5'-AAAACATATGCAACGGAGCCTGT-3') pre OXA-18Rv (5'-AAAAGGATCCTCAGAAGTTTTCCGACAGG-3') in-order-to amplify *bla*_{OXA-48} and *bla*_{OXA-18} genes, respectively. The hybrid gene of OXA-48Loop18 was constructed by overlapping PCR with partially overlapping primers. The first PCRs were done using preOXA-48A primer with P_inside_48Loop18_B (5'-ACCGTTTCGCTTTCCGATGCCAAGGGCGGCAAGGC GAAGATT GCTGGTGGGTC-3') and cloningOXA-48B primer in combination with P_inside_48Loop18_B (5'-ACCGTTTCGCTTTCCGATGCCAAGGGCGGCAAGGC GAAGATTGGCTGGTGGGTC-3'). The products of these PCR were then purified using GeneJET PCR Purification Kit (Thermo Scientific™, Montigny-le-Bretonneux, France) and mixed for being used as template for a second PCR using the primers preOXA-48A and cloningOXA-48B. The amplicons obtained in all cases were then cloned into the pCR®-Blunt II-TOPO® plasmid (Invitrogen, Illkirch, France) downstream from the pLac promoter, in the same orientation. The recombinant pTOPO-OXA plasmids were electroporated into the *E. coli* TOP10 strain. The electroporants were plated on a TSA plate containing kanamycin (50 µg/ml). The *bla*_{OXA-48Loop18} gene fragment corresponding to the mature β -lactamase was cloned into the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois, France) using the PCR generated fragment with primers INF-OXA-48Fw (5'-AAGGAGATATACATATGGTAGCAAAGGAATGGCAA G-3') and INF-OXA-48Rv (5'-GGTGGTGGTGCTCGAAGGGAATAATTTTTCTGTT TGAG-3') and the NEBuilder® HiFiDNA Assembly Cloning Kit (New England BioLabs® Inc, United Kingdom), following the manufacturer's instructions. Recombinant plasmid pET41-OXA-517 was transformed into chemocompetent *E. coli* strain BL21 (DE3).

Recombinant plasmids were extracted using the Qiagen miniprep kit and both strands of the inserts were sequenced using M13 primers, for the pCR®-Blunt II-TOPO® plasmid (Invitrogen, Illkirch, France), and T7 primers, for pET41b(+) (Novagen, VWR International, Fontenay-sous-Bois, France), with an automated sequencer (ABI

Prism 3100; Applied Biosystems). The nucleotide sequences were analyzed using software available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

β -Lactamase purification. An overnight culture of *E. coli* strain BL21 (DE3) harboring pET41b-OXA-48Loop18 was used to inoculate 2 L of LB broth containing 50 mg/L kanamycin. Bacteria were cultured at 37°C until reaching an OD of 0.6 at 600 nm. Expression of the OXA-48Loop18 was induced overnight at 25°C with 0.2 mM IPTG, as previously described (30). Cultures were centrifuged at 6000 g for 15 min and the pellets resuspended with 10 mL of Buffer A (20 mM Phosphate buffer, 175 mM SO₄K₂, 40 mM Imidazol, pH 7.4). Bacterial cells were disrupted by sonication and the bacterial debris were removed by two consecutive centrifugation steps at 10.000 g for 1 h at 4°C and 48.000 g for 1 h at 4°C. OXA-48Loop18 was purified in one step pseudo-affinity chromatography using a NTA-Nickel column (GE Healthcare, Freiburg, Germany) (30). Protein purity was estimated by SDS-PAGE, pure fractions were pooled and dialyzed against 20mM Hepes SO₄K₂ 50 mM buffer (pH 7) and concentrated by using Vivaspin® columns (GE Healthcare, Freiburg, Germany). Protein concentration was determined by Bradford Protein assay (Bio-Rad, Marnes-La-Coquette, France) (31).

Steady-state kinetic parameters. Kinetic parameters of purified OXA-48Loop18 were determined at 30°C in 100 mM sodium phosphate buffer (pH 7). The *k*_{cat} and *K*_m values were determined by analyzing hydrolysis of β -lactams under initial-rate conditions with an ULTROSPEC 2000 model UV spectrophotometer (Amersham Pharmacia Biotech) using the Eadie-Hoffstee linearization of the Michaelis-Menten equation, as previously described (32). The different β -lactams were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Protein crystallization and X-ray crystallography. Conditions for OXA-48Loop18 (26.8 mg ml⁻¹) were identified from screening stochastic crystallization conditions among the commercially available suites: Classics, AmSO₄, PEGs and PEGs II (Qiagen/NeXtal), using the Mosquito® HTS, (TTP LabTech). Screening plates hits were scaled up manually using 4 µL hanging drops versus 2.2 M ammonium sulfate, 0.2M ammonium fluoride in the reservoir at room

temperature. The tiny prismatic crystal was transferred to a cryo-protectant solution consisting of the mother liquor supplemented with 25% glycerol and flash-frozen in liquid nitrogen. Diffraction data was collected at 100 K in a nitrogen cryostream on the PROXIMA1 beamline at the SOLEIL synchrotron (Saint-Aubin, France). The data were indexed and integrated with XDS (33) via the XDSME script (<https://github.com/legrandp/xdsme> (34)).

Data scaling was performed using AIMLESS (35) from the CCP4 suite (36). Data collection and refinement statistics are given in Table 3. The structure of OXA-48Loop18 with two molecules per asymmetric unit was successfully solved using the automatic molecular replacement program *MrBUMP* (37) with OXA-48 (PDB entry 4S2K; 91% sequence identity), as a search model. The model was rebuilt manually in Coot (38) and then refined using BUSTER-TNT (39) with local noncrystallographic symmetry (NCS) restraints and a translation–libration–screw (TLS) description of B factors (40). The quality of the final refined model was assessed using MolProbity (41). Crystal structure images were generated using PyMOL (<http://www.pymol.org>) (42).

Structure analysis, docking and molecular dynamics simulations. OXA-48Loop18 structure comparison to the previously published OXA-48 structures and analysis of docking results were performed with UCSF Chimera package (43). Covalent docking experiments on OXA-48 and OXA-48Loop18 were performed using the GOLD suite (CCDC) (23). Ligand structures were generated with 3D Structure Generator CORINA Classic version 3.60 (Molecular Networks GmbH, Nuremberg, Germany). Molecular dynamics simulations of OXA-48 and OXA-48Loop18 were performed with Gromacs version 4.6 (44) using the OPLS-AA force field (45). OPLS-AA force field parameters for covalently-bound imipenem were built using a modified version of the MOL2FF package developed in our team. Hop software version 0.4.0 alpha 2 (<https://github.com/Becksteinlab/hop>) (25) was used for water molecule dynamics analysis.

PDB deposition. The crystallographic structure of OXA-48Loop18 has been deposited to the PDB, accession code 6HOO. Authors will release the atomic coordinates and experimental data upon article publication.

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Conflict of interest

The authors declare no competing interests.

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Role of the loop β 5- β 6 in the substrate specificity of OXA-48

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Abstract

Enterobacteriaceae producing OXA-48-like carbapenemases have now globally disseminated. Several variants have been reported with either amino-acid substitutions that result in similar hydrolysis profiles as for OXA-48, or with deletions located in the β 5- β 6 loop that result in the loss of carbapenem-hydrolysis and gain of expanded-spectrum cephalosporin hydrolysis. We evaluated the importance of the β 5- β 6 loop on OXA-48 hydrolysis profile was investigated using site-directed mutagenesis, replacing each amino-acid of the loop by alanine or by introducing deletions of one to six amino-acids in both directions. The resulting mutants were analyzed using phenotypical, biochemical and structural approaches. The four-amino acid deletion is characterized by loss of carbapenem-hydrolysis and gain of cephalosporin hydrolysis. Homology modelling, covalent docking, and molecular dynamics simulations of covalent complexes were used to characterize the dynamic behavior of the deletion mutants, and results offer a possible explanation for their acquired capacity to hydrolyze ceftazidime, and the observed decrease in affinity and turnover rate for imipenem. Overall, our results demonstrate the critical role of the length and amino acid composition of the β 5- β 6 loop in the substrate specificity of OXA-48.

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Introduction

Antibiotics have saved countless lives and continue to be a mainstay of therapy for bacterial infections. Even though, antimicrobial resistance (AMR) is perhaps the most alarming emerging problem of infectious diseases. Global burden of AMR is already important with 700,000 deaths/year, but the forecast for 2050, it nothing is done to cope with this problem, more than 10 million people will die/year of AMR (O'Neil report). One of the most concerning issue in antibiotic resistance is conferred by β -lactamases, which are able to hydrolyze β -lactams, including those of last generation, as carbapenems. According to Ambler classification (Ambler, 1980), there are four classes of β -lactamases, from A to D. Class D β -lactamases (DBLs), also known as oxacillinases or OXA-type β -lactamases (OXAs), present a serine located at position 70, similar to Ambler class A and class C β -lactamases, whereas class B β -lactamases are metalloenzymes, with one or two Zn^{+2} ions in the active site (Ambler, 1980; Lamotte-Brasseur et al. 1994). Among the four molecular classes of β -lactamases, class D β -lactamases are the most diverse enzymes (Naas & Nordmann, 1999). This diversity is observed at both genetic and biochemical levels, with enzymes possessing either a narrow or extended-spectrum of hydrolysis or also carbapenem-hydrolyzing capability (Poirel et al, 2010). This last group is also referred as the carbapenem-hydrolyzing class D β -lactamases (CHDLs) and has initially been found mostly in *Acinetobacter* spp. (Higgins et al, 2009; Poirel & Nordmann, 2006); OXA-48-type enzymes have increasingly been isolated among *Enterobacteriaceae* (Poirel et al, 2012). Although OXA-48 hydrolyzes penicillins at high level, it also hydrolyzes carbapenems at low level. It shows very weak activity against expanded-spectrum cephalosporins, hydrolyzes cefotaxime very poorly, but does not hydrolyze ceftazidime and cefepime (Docquier et al, 2009). OXA-48 was initially identified from a carbapenem-resistant *Klebsiella pneumoniae*

clinical isolate recovered in 2001 from Turkey (Poirel et al, 2004). Since the discovery of OXA-48, several variants have been reported that differ from OXA-48 by amino-acid substitutions or deletions

(<http://bldb.eu/BLDB.php%3fclass=D%23OXA>) (Naas et al, 2017). Even though OXA-48 and other OXA-48 like enzymes, as OXA-181 (Potron et al, 2011) and OXA-232 (Potron et al, 2013), exhibit a substrate profile including penicillins and carbapenems, but spare expanded-spectrum cephalosporins, others, like OXA-163 (Poirel et al, 2011) and OXA-405 (Dortet et al, 2015) hydrolyze expanded-spectrum cephalosporins but very weakly carbapenems.

OXA-48 3 dimensional structure is very similar to that of other DBLs despite a remarkable sequence divergence (Docquier et al, 2009). Superimposition of these 3D structures revealed small differences located mainly in the loops connecting secondary structure elements, which may vary in length and orientation. This is the case of the loop connecting the strands $\beta 5$ and $\beta 6$. This loop is close to the active site and connects two β -strands, $\beta 5$ and $\beta 6$ strands. The $\beta 5$ strand of which one includes the catalytically-relevant and conserved KTG residues and delimits one side of the active site in OXA-48 (Docquier et al, 2009). The orientation and size of the $\beta 5$ – $\beta 6$ loop is very similar in OXA-24, a CHDLs, and OXA-48, suggesting its possible involvement in the ability of some OXA variants to hydrolyze carbapenems, and therefore, alterations in this loop, for example like in OXA-163, may impact the carbapenem hydrolysis (Docquier et al, 2009; De luca et al, 2011; Stojanoski et al, 2015). Moreover, all the OXA-48-variants with non-carbapenemase activity and expanded-spectrum cephalosporins hydrolysis property, present, as only difference with OXA-48, mutations in the region of the $\beta 5$ – $\beta 6$ loop.

To provide further evidence of the role of the $\beta 5$ – $\beta 6$ loop in the hydrolysis of carbapenems by OXA-48-like carbapenemases, we investigated the contribution of each amino-acid of the loop and

	198		228	
OXA-48		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48Y211A		EANGDYIIIRAKTG ASTRIE PKIGWWVGWV EL		
OXA-48S212A		EANGDYIIIRAKTG ASTRIE PKIGWWVGWV EL		Alanine substitution
OXA-48T213A		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		mutants
OXA-48R214A		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48I215A		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48E216A		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48P217A		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
	*****	*****		
	198		228	
OXA-48		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔY		EANGDYIIIRAKTG STRIE PKIGWWVGWV EL		Right direction
OXA-48ΔYS		EANGDYIIIRAKTG STRIE PKIGWWVGWV EL		group
OXA-48ΔYST		EANGDYIIIRAKTG STRIE PKIGWWVGWV EL		
OXA-48ΔYSTR		EANGDYIIIRAKTG STRIE PKIGWWVGWV EL		
OXA-48ΔYSTRI		EANGDYIIIRAKTG STRIE PKIGWWVGWV EL		
OXA-48ΔYSTRIE		EANGDYIIIRAKTG STRIE PKIGWWVGWV EL		
	*****	*****		
	198		228	
OXA-48		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔP		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		Deletion mutants
OXA-48ΔEP		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔIEP		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔRIEP		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔTRIEP		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔASTRIE		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔYSTRIE		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
	*****	*****		
	198		228	
OXA-48		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔP		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		Left direction
OXA-48ΔEP		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		group
OXA-48ΔIEP		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔRIEP		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔTRIEP		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔASTRIE		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
OXA-48ΔYSTRIE		EANGDYIIIRAKTG YSTRIE PKIGWWVGWV EL		
	*****	*****		

Figure 1. Amino acids sequences comparison of the β5-β6 loop region in different mutants. In alanine substitution mutants, the substitutions are shown in grey. In deletion mutants, the deletion are represented as "-". The β5-β6 loop is represented in bold letters.

of amino-acid deletions starting on both ends of the β5-β6 loop in respect to carbapenem, and expanded-spectrum cephalosporin hydrolysis. Thus, twenty mutants of OXA-48 were analyzed in this study and compared to OXA-48 (Figure 1).

Results

MIC results of OXA-48 mutants expressed in *E. coli*.

To determine the effect of the alanine substitutions of each amino-acid residue of the β5-β6 loop, MIC values of *E. coli* TOP10 (pTOPO-OXA-48-MUT1-20) were compared to those of *E. coli* TOP10 (pTOPO-OXA-48). Overall the impact on MICs for the different substrates tested was minor. MIC values for temocillin and amoxicillin were unaffected, while, MICs values for ceftazidime of *E. coli* TOP10 (pTOPO-OXA-48 Y211 and *E. coli* TOP10 (pTOPO-OXA-48 P217A) were ~ 3-fold higher as compared to that of *E. coli* TOP10 (pTOPO-OXA-48) (Table 1) . On the opposite, MICs

values for imipenem were ~3-fold lower for *E. coli* TOP10 (pTOPO-OXA-48 R214A) (Table 1). The most significant differences in MICs were observed with the deletion mutants. Indeed, MIC values for imipenem, ceftazidime, aztreonam and temocillin were remarkably affected. Starting from the N-terminus of the β5-β6 loop, MICs for Imipenem and temocillin decreased along with the size of the deletion, and those of ceftazidime increased with a maximum value for deletions of 4 amino-acids (OXA-48ΔYSTR) with MICs ~ 32-fold compared to OXA-48 producing strain. Similar results were obtained with deletions starting from the right side, with the exception of ΔP that displayed significant carbapenem and expanded spectrum cephalosporin hydrolysis (Table 1), maximum ceftazidime hydrolysis was obtained with three amino acid deletions (OXA-48ΔIEP producer) ~

Table 1 MICs of β -lactams for *E. coli* TOP 10(p-TOPO-OXA-48), its variants and *E. coli* TOP 10.

<i>E. coli</i> Top10 (p-TOPO-(Mutant))	MIC (mg/L)											
	Ampicillin	Ampicillin + CLA ^a	Piperacillin	Piperacillin + TZB ^b	Temocillin	Cefotaxime	Ceftazidime	Cefepime	Aztreonam	Imipenem	Meropenem	Ertapenem
(OXA-48)	>256	>256	>256	>256	>1024	0.75	0.19	0.19	0.047	0.75	0.25	0.25
(OXA-48 Y-211-A)	>256	>256	>256	>256	>1024	0.094	0.5	0.094	0.25	0.5	0.125	0.25
(OXA-48 S-212-A)	>256	>256	>256	>256	>1024	0.19	0.25	0.094	0.047	1	0.125	0.25
(OXA-48 T-213-A)	>256	>256	>256	>256	>1024	1	0.25	0.5	0.047	0.75	0.19	0.25
(OXA-48 R-214-A)	>256	>256	>256	>256	>1024	0.38	0.125	0.0125	0.047	0.25	0.125	0.25
(OXA-48 I-215-A)	>256	>256	>256	>256	>1024	0.25	0.19	0.064	0.047	0.75	0.19	0.25
(OXA-48 E-216-A)	>256	>256	>256	>256	>1024	1	0.19	0.25	0.047	0.75	0.19	0.38
(OXA-48 P-217-A)	>256	>256	>256	>256	>1024	1	0.5	0.38	0.047	0.75	0.25	0.25
(OXA-48 Δ Y)	>256	>256	>256	>256	>1024	0.064	1	0.064	3	0.5	0.125	0.19
(OXA-48 Δ YS)	>256	>256	>256	>256	96	0.19	3	0.75	1.5	0.25	0.032	0.032
(OXA-48 Δ YST)	>256	>256	>256	>256	64	0.19	0.25	0.094	3	0.25	0.032	0.047
(OXA-48 Δ YSTR)	>256	>256	>256	>256	12	0.125	6	0.5	0.75	0.19	0.032	0.023
(OXA-48 Δ YSTRI)	3	3	2	2	8	0.032	0.125	0.032	0.032	0.125	0.016	0.002
(OXA-48 Δ YSTRIE)	3	3	2	2	8	0.047	0.25	0.032	0.047	0.19	0.016	0.002
(OXA-48 Δ P)	>256	>256	>256	>256	>1024	0.38	3	0.25	0.125	0.75	0.064	0.094
(OXA-48 Δ EP)	>256	>256	>256	>256	192	0.125	0.5	0.064	0.125	0.75	0.064	0.094
(OXA-48 Δ IEP)	>256	>256	>256	>256	64	0.25	8	0.25	0.19	0.5	0.064	0.064
(OXA-48 Δ RIEP)	>256	>256	>256	>256	16	1.5	6	2	2	0.25	0.032	0.023
(OXA-48 Δ TRIEP)	>256	>256	96	96	16	1.5	6	2	1.5	0.25	0.032	0.023
(OXA-48 Δ STRIEP)	3	3	1.5	1.5	8	0.032	0.125	0.032	0.047	0.19	0.016	0.002
(OXA-48 Δ YSTRIEP)	4	4	1.5	1.5	8	0.047	0.19	0.032	0.047	0.19	0.016	0.002
<i>E. coli</i> TOP 10	2	2	1.5	1	4	0.06	0.12	0.023	0.047	0.25	0.016	0.003

^aCLA, clavulanic acid fixed concentration of 2mg/L.

^bTZB, tazobactam at fixed concentration of 4 mg/L.

42-fold higher as compared to OXA-48-producer. MIC values for aztreonam are low for OXA-48 producers (0.047 mg/L) while most of the deletion mutants displayed higher MICs, with the highest values obtained for the four amino-acid deletion mutant in the left direction (OXA-48 Δ RIEP), $\sim$ 43-fold increase, and for the three amino-acid deletion in the right direction (OXA-48 Δ Y and OXA-48 Δ YST), $\sim$ 64-fold bigger than the OXA-48 recombinant strain.

kinetic parameters determination

In order to examine the different substrate specificities of the different deletion mutants and compare them with the ones of OXA-48, steady-state kinetic parameters were determined for several β -lactams. The highest catalytic efficiencies (k_{cat}/K_m) for imipenem hydrolysis were observed in OXA-48 and in OXA-48 Δ P (369 and 386 mM $^{-1}$ /s $^{-1}$, respectively) (Table 2), while as the deletion increased, the smaller were the k_{cat}/K_m values. This trend was noticed in both groups of deleted mutants (left and right directions) and it was in accordance with the MIC data presented previously.

Another interesting result was observed in aztreonam and ceftazidime hydrolysis. In both cases, OXA-48 was not able to hydrolyze these β -lactams, whereas almost all of the deleted mutants could hydrolyze aztreonam and ceftazidime. Concerning aztreonam hydrolysis, in the right direction deletion mutants group, the highest catalytic efficiency value correspond to OXA-48 Δ YST (3.1 mM $^{-1}$ /s $^{-1}$). In the left direction group, OXA-48 Δ RIEP and OXA-48 Δ TRIEP presented the highest k_{cat}/K_m values (9.0 mM $^{-1}$ /s $^{-1}$ and 7.7 mM $^{-1}$ /s $^{-1}$). Although OXA-48 Δ RIEP showed a K_m value of 451 μ M against 115 μ M corresponding to OXA-48 Δ TRIEP, the turnover number of OXA-48 Δ RIEP was $\sim$ 5-fold higher than the one for OXA-48 Δ TRIEP (4.1 s $^{-1}$ and 0.88 s $^{-1}$, respectively) (Table 2). Regarding ceftazidime, in the deletions from the left direction, OXA-48 Δ P,

OXA-48 Δ RIEP and OXA-48 Δ TRIEP showed the highest k_{cat}/K_m values () and presented high MICs, although they were not the highest ones. The highest MIC corresponds to OXA-48 Δ IEP, which surprisingly has a $k_{cat}/K_m \sim$ 6-fold smaller than the one for OXA-48 Δ P (1.24 mM $^{-1}$ /s $^{-1}$). In the deletions from the right direction, OXA-48 Δ YSTR and OXA-48 Δ YS presented the highest catalytic efficiencies, 0.36 mM $^{-1}$ /s $^{-1}$ and 0.31 mM $^{-1}$ /s $^{-1}$ respectively, which were consistent with the MIC values observed.

Analyzing the hydrolysis of ampicillin, all the deletions from the left direction were able to hydrolyze it but with a significant difference in the k_{cat} , c.a. 15-fold lower than that of OXA-48, which had an impact in the k_{cat}/K_m values of c.a. 12-fold. Deletion mutants OXA-48 Δ IEP, OXA-48 Δ RIEP and OXA-48 Δ TRIEP, were not able to hydrolyze ampicillin while their MICs were high. In order to see whether ampicillin may act as an inhibitor on these mutants, we determined IC₅₀. (Table 3). Indeed, ampicillin had significant inhibitory activity of these mutants. Similar results were obtained for temocillin. In the deletion mutants from the left direction, only OXA-48 Δ P could hydrolyze it, and this hydrolysis was with the same catalytic efficiency as of OXA-48. The rest of the mutants of this group were inhibited by temocillin, with IC₅₀ values increasing with the length of the deletion (Table 3).

Table 3 IC₅₀ values for OXA-48 deletion mutants for ampicillin and temocillin.

Mutant	IC ₅₀ (μ M)	
	Ampicillin	Temocillin
OXA-48 Δ P	H	H
OXA-48 Δ EP	H	0.15
OXA-48 Δ IEP	15	1
OXA-48 Δ RIEP	0.07	6
OXA-48 Δ TRIEP	8	30
OXA-48 Δ STRIEP	H	240
OXA-48 Δ YSTRIEP	H	1500

H, hydrolysis.
Values calculated on the basis of three independent experiments

Table 2. Steady-state kinetic parameters of OXA-48 and its mutants towards representative β -lactam substrates.

Enzyme	$K_m(\mu\text{M})$						$k_{\text{cat}}(\text{s}^{-1})$						$k_{\text{cat}}/K_m (\text{mM}^{-1}/\text{s}^{-1})$					
	Ampicillin	Aztreonam	Cefalotin	Ceftazidime	Imipenem	Temocillin	Ampicillin	Aztreonam	Cefalotin	Ceftazidime	Imipenem	Temocillin	Ampicillin	Aztreonam	Cefalotin	Ceftazidime	Imipenem	Temocillin
OXA-48	395	NH	195	NH	13	45	955	NH	44	NH	4.8	0.30	2388	NH	226	NH	369	6.7
OXA-48 ΔY	117	>1000	688	>1000	172	350	6.9	>2.1	6.4	>0.17	0.64	0.06	59	0.48	9.3	0.02	3.7	0.17
OXA-48 ΔYS	14	665	756	281	>1000	>1000	2.7	0.76	12	0.09	>0.98	>0.47	185	1.1	16	0.31	0.39	0.07
OXA-48 ΔYST	784	667	353	>1000	149	762	62	2.1	0.62	>0.04	0.08	0.14	79	3.1	1.8	0.01	0.52	0.18
OXA-48 $\Delta YSTR$	312	431	177	338	>1000	>1000	54	0.36	1.4	0.12	>0.06	>0.41	172	0.84	7.8	0.36	0.02	0.18
OXA-48 $\Delta YSTRI$	>1000	>1000	>1000	NH	>1000	NH	>0.41	>0.16	>0.03	NH	>0.02	NH	0.04	0.03	0.01	NH	0.02	NH
OXA-48 $\Delta YSTRIE$	NH	NH	NH	NH	>1000	NH	NH	NH	NH	NH	>0.06	NH	NH	NH	NH	NH	0.02	NH
OXA-48 ΔP	264	>1000	175	42	8	530	99	>0.35	6.8	0.05	3	3.1	374	0.32	39	1.2	386	5.9
OXA-48 ΔEP	167	763	274	624	71	NH	75	0.59	17	0.10	2.2	NH	451	0.77	64	0.16	31	NH
OXA-48 ΔIEP	NH	584	213	>1000	469	NH	NH	0.30	12	>0.25	2.9	NH	NH	0.51	55	0.22	6.2	NH
OXA-48 $\Delta RIEP$	NH	451	80	648	>1000	NH	NH	4.1	18	0.61	>0.71	NH	NH	9.0	222	0.94	0.27	NH
OXA-48 $\Delta TRIEP$	NH	115	33	339	>1000	NH	NH	0.88	7.63	0.25	>0.21	NH	NH	7.7	234	0.74	0.14	NH
OXA-48 $\Delta STRIEP$	373	NH	175	>1000	NH	NH	1.3	NH	0.08	>0.10	NH	NH	3.5	NH	0.46	0.06	NH	NH
OXA-48 $\Delta YSTRIEP$	>1000	NH	548	>1000	813	NH	>0.11	NH	0.007	>0.12	0.17	NH	0.08	NH	0.01	0.11	0.21	NH
OXA-48 P-217-A	256	ND	ND	324	14	ND	69	ND	ND	0.02	3	ND	268	ND	ND	0.07	214	ND

ND, not determined; NH, not detectable hydrolysis was observed with 5 μM purified enzyme and up to 1000 μM substrate.

Values calculated on the basis of three independent experiments. SDs were below 10%.

Data for OXA-48 are from Docquier *et al.*

The particular case of OXA-48 Δ P

One particular deletion mutant, OXA-48 Δ P, drew our attention. Although it presented only one amino-acid deletion, P217, the profile of hydrolysis was very interesting. This mutant could hydrolyze imipenem with the same catalytic efficiency as OXA-48 and at the same time, presented the highest value of hydrolysis of ceftazidime. For this reason, we decided to investigate more deeply the role of this proline at position 217. We determined the kinetic parameters of the mutant OXA-48 P217A to analyze more in detail the effect of the substitution of the P217 (Table 2). We observed that regarding ampicillin the activity was similar to the one of OXA-48 Δ P, and the difference with OXA-48 was in the turnover number, 10-fold lower than that of OXA-48. Even though, the hydrolysis of amoxicillin presented high values of k_{cat}/K_m , what was in agreement with the observed in the MIC values (Table 1). In the case of ceftazidime, the substitution of the P217 produced a reduction in the K_m compared to OXA-48. The mutants OXA-48 P217A and OXA-48 Δ P differ in the K_m values, OXA-48- Δ P showing the lowest one. No significant differences were observed in the turnover number of OXA-48 P217A and OXA-48- Δ P, raising the possibility that the P217 participates in the binding of the ceftazidime, but not in its hydrolysis. No significant differences were observed with imipenem, the three enzymes (OXA-48, OXA-48 P217A and OXA-48- Δ P mutants) hydrolyze it with very similar k_{cat}/K_m values, giving the idea that the P217 did not interfere with the binding of imipenem in the active site.

Imipenem docking on OXA-48

Imipenem was covalently docked on the available structure of OXA-48 (PDB code 4S2P) (King et al, 2015). Docking results show imipenem in a conformation similar to those observed in the structure of OXA-48 co-crystallized with imipenem (PDB code 5QB4) (Akhter et al, 2018), with the carbonyl oxygen buried in the oxanion hole, the R1 6 α -hydroxyethyl substituent

positioned inside the cavity delimited by KCX73, W105, V120, L158 and T213, with the methyl group pointing towards L158, and the C3 carboxylate making hydrogen bonds with R250 and T209 (Fig 2). The main difference is observed for the R2 substituent, which points upwards, making a hydrogen bond with T104. In the 5QB4 crystal structure, the R2 substituent is making non-polar contacts with Y211 and L247, but adopting a slightly different conformation in each of the four crystal monomers, suggesting that its conformation in the covalent complex in solution might also be variable.

Molecular dynamics simulations of imipenem complexes

In order to look into how the loop mutations affect the imipenem turnover rate, molecular dynamics (MD) simulations of the covalent complexes were performed. Homology model structures for the mutants were built based on the OXA-48 structure with imipenem docked inside, as a starting point for the simulations. For the sake of clarity, in the following sections residue numbers are kept according to the OXA-48 numbering, disregarding the shift in position in the primary sequence caused by each deletion.

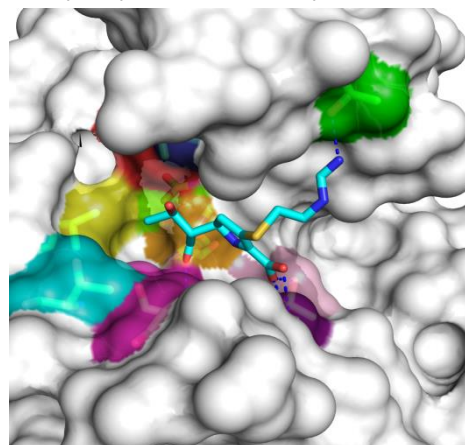


Figure 2. Docking of imipenem on the OXA-48 structure. Covalent complex of imipenem and OXA-48, created by covalent docking on the PDB 4S2P structure. The conformation adopted by imipenem is notably similar to the one observed in the PDB 5QB4 structure, with the exception of the R2 substituent orientation. Surface colored according to residue: orange: S70, light green: KCX73, green: T104, blue: W105, red: V120, yellow: L158, cyan: T213, pink: T209, fuchsia: Y211, purple: R250. Hydrogen bonds are depicted in dashed blue lines.

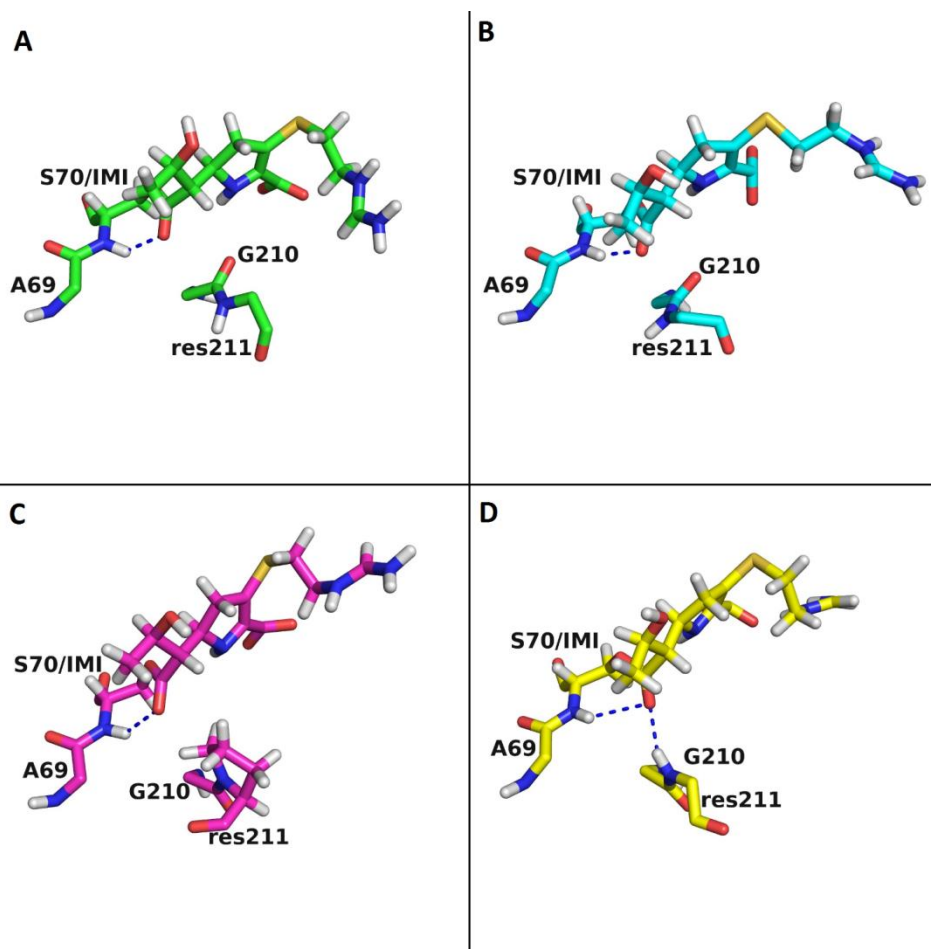


Figure 3. Oxyanion hole disruption for the last 4 N-terminus deletion mutants. Representative structures from the imipenem complex MD simulations show how the oxyanion hole is disrupted in mutants OXA-48ΔYSTR (A), OXA-48ΔYSTRI (B) and OXA-48ΔYSTRIE (C), but is restored in OXA-48ΔYSTRIEP (D). Hydrogen bonds are depicted as dashed blue lines.

The initial structures were subjected to energy minimization, equilibration with restraints on the heavy atoms, and 10 ns production MD simulations. Trajectories were then centered and fitted on C α atoms, clustered, and the most representative structure for the simulation was extracted.

Results show diversity in the conformation of the β 5- β 6 loop and the active site surface corresponding to it, as well as changes in the hydrophobicity (Fig. S1-S14).

These changes on the surface seem to be more abrupt when starting deletions from the N-terminus of the β 5- β 6 loop. Already the first deletion, OXA-48ΔY (Fig. S2), removes Y211, shifting S212, T213, R214 and I215 towards the active site. The β 5- β 6 loop acquires a conformation with a more abrupt turn towards

the Ω -loop. The peptide bond between S212 and T213 is inverted, with the S212 sidechain replacing the hydrogen bond with the β 6 strand. T213 and R214 are displaced outwards by the loop turn, by breaking in the meantime the R214-D159 salt bridge, and I215 is contacting L158 from beneath. These changes seem to disrupt the hydrophobic pocket formed between L158, T213 and Y221 sidechains (Docquier et al, 2009), both due to the outwards displacement and the position shift of R214 towards the active site. The following N-terminus deletion mutant, OXA-48ΔYS (Fig. S3), seems to partially correct the abrupt displacement of the loop caused for OXA-48ΔY, while simultaneously shifting R214 closer into the active site cavity, partially filling the space the R2 substituent of imipenem is occupying in the 5QB4 structure, and also making

for a smaller cavity. The OXA-48 Δ YST (Fig. S4) mutant places R214 in the position of Y211, adopting the same orientation, widening the active site cavity again. Similar or more abrupt disruptions are observed on position 211 for the following N-terminus deletion mutants. Further deletions (OXA-48 Δ YSTR, OXA-48 Δ YSTRI) appear

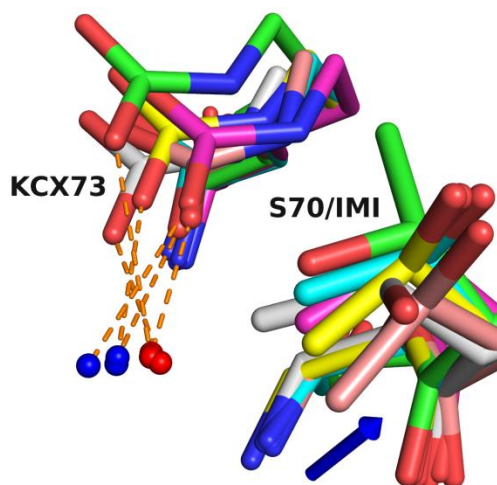


Figure 4. Hydrolytic water position inside the active site cavity. Hop analysis results showing the average position of the hydrolytic water molecule inside the active site cavity. OXA-48 and mutants maintaining good turnover rates, such as OXA-48 Δ P and OXA-48 Δ EP, show water molecule positions (red spheres) closer to the scissile bond (pointed at by the blue arrow) than mutants with bad turnover rates, such as OXA-48 Δ Y, OXA-48 Δ YS and OXA-48 Δ YSTRIEP (blue spheres). Only Imipenem, S70 and KCX73 are depicted for clarity. Hydrogen bonds are depicted as dashed orange lines.

to completely disrupt the oxyanion hole by twisting the β 5 strand backbone at position 211 (Fig. S5 and S6; Fig. 3). OXA-48 Δ YSTRIE (Fig. S7) places P217 in position 211, where its secondary amine has no hydrogen to contribute to forming the oxyanion hole (Fig. 3). Deletion of the whole β 5- β 6 loop in mutant OXA-48 Δ YSTRIEP (Fig. S8) places K218 at position 211, with its sidechain elongated outwards, making for a polar wall where T213 usually forms the non-polar patch together with L158. Notably, the backbone of position 211 (here K218) adopts once again a conformation where the oxyanion hole may exist (Fig. 3).

In contrast, C-terminus deletions seem to make a more gradual disruption of the original OXA-48 β 5- β 6 loop conformation and cavity surface. The first 3 deletions (OXA-48 Δ P, OXA-48 Δ EP, OXA-48 Δ IEP) appear to have relatively little effect on the surface conformation of the loop C-terminus (Fig. S9, S10 and S11). The shape of the loop remains similar to the original one. Y211 and S212 maintain a conformation and position close to the original one too. A non-polar concavity between L158, T213 and Y211 is still observed, and R214 still interacts through a hydrogen bond with D159. These are the only three mutants that still make hydrogen bonds between these residues, conserving them throughout the 10 ns simulation. Coincidentally, they are also the three mutants displaying the smallest loss in k_{cat} for imipenem. Further deletions (Fig. S12, S13 and S14) seem to distort more the conformation of the end of the β 5 strand, displacing Y211 from its native conformation, and twisting its backbone, which may distort the still existing oxyanion hole, unlike for the N-terminus deletion mutants.

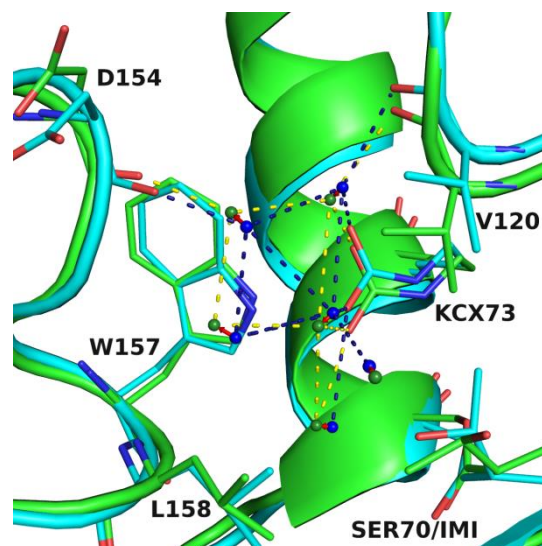


Figure 5. Water network displacement in the OXA-48 deletion mutants. Water network inside the active site cavity of OXA-48 (cyan sticks, blue spheres, blue dashed lines) and OXA-48 Δ YSTRIEP (green sticks, green spheres, yellow dashed lines) superposed. Hop analysis results show the average position of water molecules in the active site cavity to be shifted for all mutant structures in the same manner. Only imipenem, S70, KCX73, V120, D154, W157 and L158 are depicted as sticks for clarity.

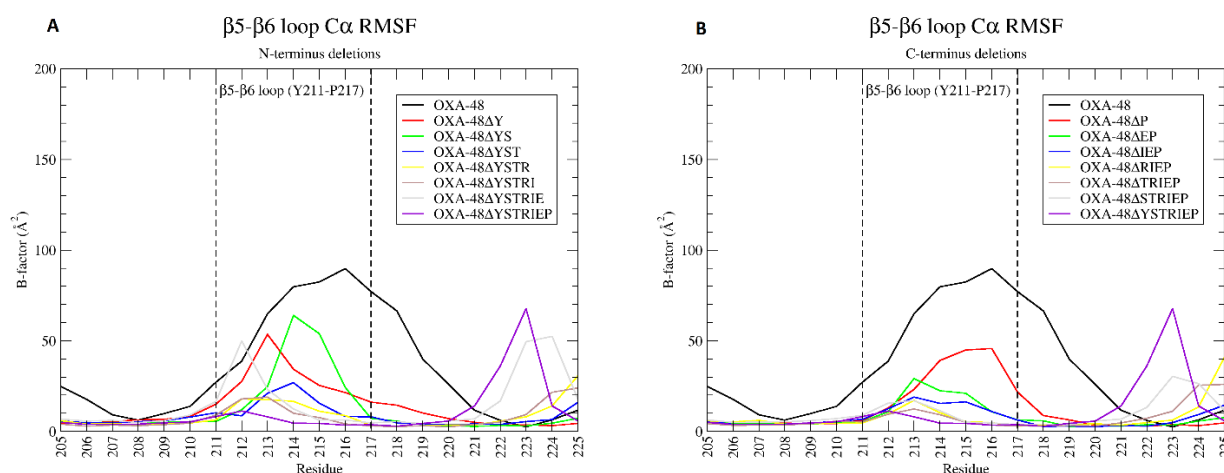


Figure 6. β5-β6 loop flexibility for OXA-48 and deletion mutants. Cα B-factor determination for the MD simulations shows the β5-β6 loop of deletion mutants to be less flexible than that of OXA-48, for both N-terminus and C-terminus deletion mutants

Dynamic water network analysis by HOP

To explore whether the position of the hydrolytic water was also influenced by the loop deletions, Hop software (Beckstein et al, 2009) was used to analyse the MD simulations. The results show that differences in the distance from the water molecule to the scissile bond exist, with distances around 3.7 Å for OXA-48 and mutants that maintain k_{cat} values above 2.0 (e.g. OXA-48ΔP and OXA-48ΔEP), and distances longer than 4 Å for mutants with k_{cat} values below 1.0 (e.g. OXA-48ΔY, OXA-48ΔYS or OXA-48ΔYSTRIEP) (Fig. 4). The lower k_{cat} values may also be correlated with the disposition of the water molecules network in the active site cavity. Compared to OXA-48, the water network seems to be slightly displaced farther from the scissile bond for all mutants (Fig. 5).

Ceftazidime docking on OXA-48 and mutant structures

Covalent docking of ceftazidime on the active site cavity of OXA-48 showed conformations that either had severe clashes between the aminothiazole ring and L158 and the whole R1 substituent and Y211, S212, T213 and R214 in the β5-β6 loop, or were incoherent with the canonical binding orientation of cephalosporins on β-lactamases, with the R1 substituent oriented

towards K208 and the dihydrothiazine ring towards the Ω-loop. This kind of results are obtained even if using the OXA-48/avibactam complex structure (PDB code 4S2N), with a slightly widened active site cavity. This supports the idea that the cavity of OXA-48 is too narrow to bind bulky cephalosporins like ceftazidime. Covalent docking of ceftazidime on the homology models presents the same kind of clashes as for OXA-48. These results were expected, as Modeller tries to respect the position of Cα atoms when generating homology models.

Superposition of the OXA-225/ceftazidime complex structure on cluster structures from MD simulations of mutant models

The β5-β6 loop flexibility in the MD simulations was examined, as it might explain their acquired capacity to bind and hydrolyze ceftazidime. However, RMSF calculations reveal the β5-β6 loop of the mutants not to be more flexible than that of OXA-48 (Fig. 6). To investigate whether the mutant loops could adopt different conformations in spite of not being more flexible, MD trajectories for the imipenem complexes were clustered and central structures extracted for each cluster. For OXA-48 3 clusters were obtained, one major, representing more than 98 % of frames, and two very minor, each one representing less than 1 % of the total number of

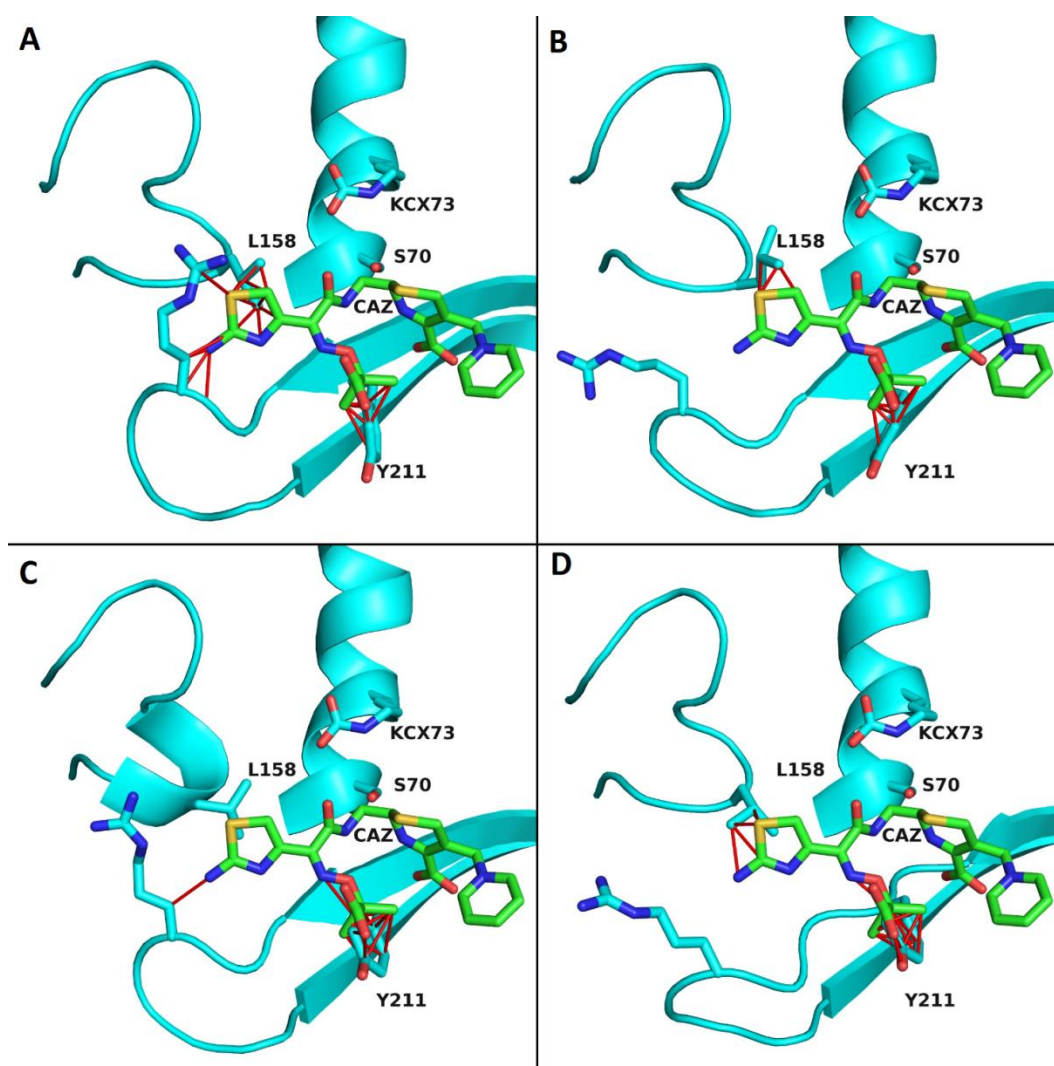


Figure 7. Ceftazidime overlaps with OXA-48 and OXA-48 Δ P. Superposition of the OXA-225/ceftazidime complex structure (PDB code 4X55) with the central structure for the single OXA-48 MD cluster (A) and the three most populated clusters of OXA-48 Δ P (B,C,D). Clashes are observed between ceftazidime and L158, R214, and Y211 for both structures, but in the case of OXA-48 Δ P at least one of the representative structures shows non-existent or smaller overlaps with ceftazidime. For clarity, only α 3 helix, β 5 and β 6 strands, and Ω and β 5- β 6 loops are depicted as ribbon, and S70, KCX73, L158, Y211, and R214 as sticks. Red lines denote overlaps.

frames. The conformation adopted by this loop corresponds quite well with the one observed in crystal structures. In contrast, for the OXA-48 Δ P MD simulation 11 clusters were obtained, 6 of being significant. Similar results were obtained for the other mutants, with most of them having 3 or more clusters containing a significant number of frames. The most representative structure for each cluster was obtained and superposed on the ceftazidime/OXA-225 complex structure (PDB code 4X55) (Mitchell et al, 2015). For the single OXA-48 cluster representative structure, severe

clashes would occur between the R1 substituent and the L158 sidechain, but also with the R214 and Y211 sidechains (Fig. 7A). In the case of OXA-48 Δ P, the same kind of overlaps would occur with L158, Y211 and R214, but for each one of these at least one of the cluster representative structures would present a sidechain conformation causing only slight clashes (for Y211) or no clashes at all (for L158 and R214) (Fig. 7B,C,D). The L158 C- α -C β -C γ dihedral angle distribution was determined for each MD simulation. For OXA-48 and most mutants, only one conformer is populated

L158 chi1 dihedral distribution

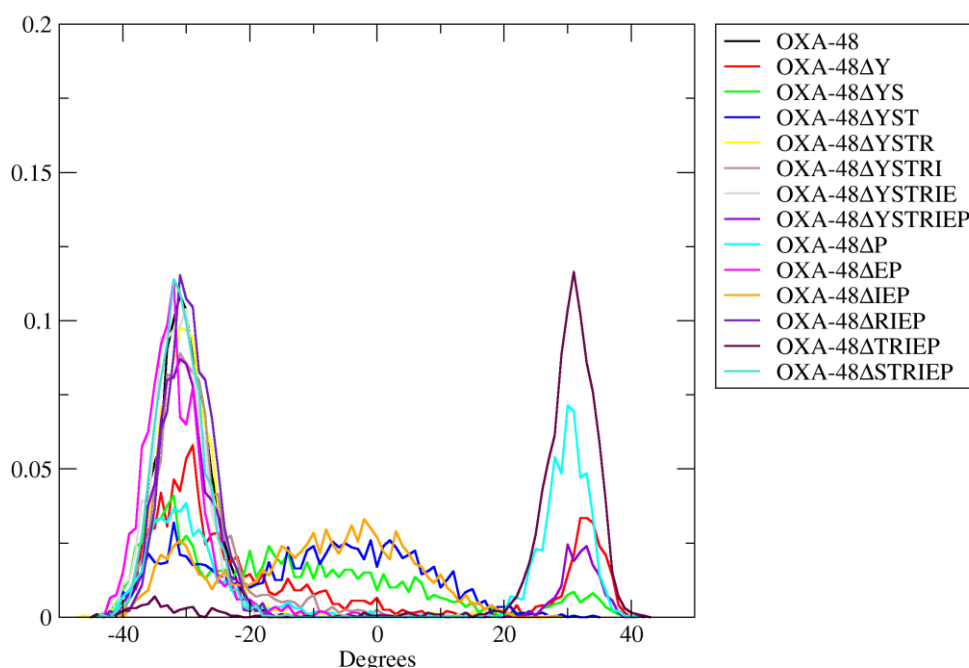


Figure 8. L158 C- α -C β -C γ dihedral angle distribution during the MD simulation for OXA-48 and deletion mutants. OXA-48 and most deletion mutant structures populate only one of the main three possible chi1 angles, whereas OXA-48 Δ P, OXA-48 Δ TRIEP, OXA-48 Δ Y, OXA-48 Δ YS and OXA-48 Δ YSTRIEP populate also a second conformation that would pose less steric impediment for the binding of ceftazidime.

throughout the 10 ns simulations, which would cause overlaps with ceftazidime (Fig. 8). A second conformation is equally populated for the OXA-48 Δ P and OXA-48 Δ TRIEP mutants, and also for OXA-48 Δ Y, OXA-48 Δ YS and OXA-48 Δ YSTRIEP, but to a lesser extent. This second conformer for L158 would cause no clashes with ceftazidime. These results suggest that the mutant structures, while not presenting a more flexible β 5- β 6 loop, may still be able to adopt a conformation competent for ceftazidime hydrolysis, thus explaining their hydrolysis spectrum expansion.

Discussion

Hydrolysis of ceftazidime, aztreonam and carbapenems by an oxacillinase were previously reported in class D β -lactamase of *Acinetobacter*

baumanni but never in OXA-48 family (Mitchell et al, 2015; Kaitany et al, 2013). Moreover, it was previously reported the critical role of the residues of the β 5- β 6 loop in determining the behavior of the enzyme toward carbapenem substrates, although not in affecting its ability to hydrolyze other substrates (like penicillins or cephalosporins). (Docquier et al, 2009; De luca et al, 2011). Here we present evidence for the participation of the residues of the β 5- β 6 loop in the substrate specificity of OXA-48, not only with carbapenems but also with expanded-spectrum cephalosporins and aztreonam. The R214 is a very important residue that participates in the hydrolysis of carbapenems. This residue defines the active cavity of OXA-48 and is also responsible for the close conformation of the β 5- β 6 loop

forming a salt bridge with D159 of the Ω loop (Docquier et al, 2009). When R214 was substituted by Ala, the hydrolysis of carbapenems was affected, which was reflected in the decrease of MIC values. Notably, the same result was observed in the natural OXA-48-variant, OXA-244 (Oteo et al, 2013), although the mutation is R214G instead of an alanine substitution, the hydrolysis of carbapenems is affected, being weaker than the one produced by OXA-48.

Another interesting residue is the P217. Natural variants of OXA-23 and OXA-24/40 in this position were reported, named OXA-225 and OXA-160 respectively, containing a substitution Pro to Ser in the homologous positions of the P217 of the $\beta 5$ – $\beta 6$ loop of OXA-48. In both mutants this substitution imparts hydrolytic activity against late generations of cephalosporins and aztreonam, without losing the carbapenemase activity (Mitchell et al, 2015). The deviation of the loop therefore provides a simple explanation for the tighter affinity of these drugs for OXA-160 (and OXA-225) compared to OXA-24/40 (and OXA-23) (Mitchell et al, 2015). In our analysis, we observed the enlargement of the spectrum of action in both cases, in the P217A substitution and also in the OXA-48- Δ P mutant, while maintaining the carbapenemase activity.

One notable common characteristic of all OXA-48 variants that hydrolyze expanded-spectrum cephalosporins (Oueslati et al, 2015), but not carbapenems, is the deletion of four amino acids of the $\beta 5$ – $\beta 6$ loop. The structural analysis of OXA-163 (Stojanoski et al, 2015) revealed that the $\beta 5$ – $\beta 6$ loop is shorter because of the 214RIEP217 deletion that results in an expanded active-site cavity compared to that of OXA-48. Several spatial modifications in the active site of OXA-163 expand the active site and rearrange the inter-residue interaction network. The larger active site is consistent with improved accommodation of ceftazidime and the loss of critical interactions with carbapenem substrates, resulting in an altered substrate profile for OXA-163 (Stojanoski et al, 2015). The analysis of our

deleted mutants shows clearly how the shortening of the $\beta 5$ – $\beta 6$ loop reduce the coefficient of activity of imipenem. It is important to point out that, even though the coefficient is reduced in almost all the mutants, the changes are more abrupt when the deletions start in Y211. When the deletions start in the P217 the decrease is more gradual. The decrease in the activity against imipenem seem to be in relation with the turnover number, and again, the mutants with deletions starting from the Y211 present k_{cat} values markedly different from that of OXA-48, while with the mutants with deletions from P217 the k_{cat} values decrease slowly. Moreover, it was previously reported that carbapenemase activity of OXA-163 is attenuated largely because of a reduction in the turnover number, while the affinity is very similar to the affinity of OXA-48 for carbapenems (Stojanoski et al, 2015).

On the contrary, in all the deleted mutants the increase in the activity against ceftazidime is at the expense of the increase of the affinity (K_m), the mutants with the deletion starting in the P217 being in this case the most affected. Something to highlight is that in both groups of deleted mutants, the maximum ceftazidimase activity, with no carbapenemase activity, is reached only when the R214 is deleted.

From a structural point of view, the $\beta 5$ – $\beta 6$ loop OXA-48 appears to be quite stable, apparently adopting a single conformation. This conformation would be determined by its short length, relative to other OXA enzymes, the presence of P217, which may turn it more rigid or restrain its freedom to change conformation, and the presence of R214, connecting the $\beta 5$ – $\beta 6$ loop to the Ω -loop via a salt bridge with D159 (Docquier et al, 2009; De Luca et al, 2011; Mitchell et al, 2015). The conformation of the loop adopted by the OXA-48 Δ Y, with a more closed turn towards the Ω -loop, may be a consequence of shortening the loop while still keeping the strain imposed by P217. Further deletions (OXA-48 Δ YSTR, OXA-48 Δ YSTR1), while

still keeping P217, appear to completely disrupt the oxyanion hole by twisting the $\beta 5$ strand backbone at position 211. Notably, for OXA-48 Δ YSTRIEP, after deletion of P217, the backbone of position 211 (now occupied by K218) adopts once again a conformation where the oxyanion hole may exist. This, and the presence of an oxyanion hole for all of the C-terminus mutants, suggests that the distortion of the oxyanion hole was heavily influenced by the presence of P217. These conformational changes at the end of the $\beta 5$ strand may explain the determined turnover rates for these mutants, with almost non-existent k_{cat} values for OXA-48 Δ YSTR, OXA-48 Δ YSTRI and OXA-48 Δ YSTRIE, and then slightly higher values for OXA-48 Δ YSTRIEP.

In contrast, C-terminus deletions, starting by deleting P217, seem to make a more gradual disruption of the original OXA-48 $\beta 5$ - $\beta 6$ loop conformation and cavity surface. Further C-terminus deletions, however, seem to pose a bigger strain on the loop and distort more the conformation of the end of the $\beta 5$ strand. The native conformation of Y211 is affected, and its backbone twists slightly back, affecting the oxyanion hole, which is still present.

The capacity of OXA-48 to hydrolyze imipenem, and its inability to bind ceftazidime, seem to be influenced by the sequence and conformation of this loop. The conformation adopted by the loop backbone, and the Y211, T213 and R214 sidechains seem to be a steric impediment for the binding of ceftazidime. The MIC values for the alanine substitution mutants suggest that the biggest impediments would come from Y211 and the general conformation of the loop is imposed by P217. The sidechain of the L158 residue, present on the Ω -loop, may also be a steric impediment for ceftazidime (Mitchell et al, 2015), and the conformation that this residue can adopt may also depend on the interaction with the adjacent $\beta 5$ - $\beta 6$ loop.

Mutations involving the residues at positions 211-213 and their conformations inside the active site cavity may also affect the affinity

for imipenem, whose R2 substituent makes non-polar interactions with the Y211 sidechain (Akhter et al, 2018). The more gradual increase in K_m for imipenem observed for the C-terminus deletion mutants, which affects the N-terminus of the loop to a lesser extent, and the abrupt increase and decrease observed for the first 3 N-terminus deletion mutants seem to corroborate this idea.

Regarding the turnover rate for imipenem, T213, whose position is influenced by the conformation adopted by the $\beta 5$ - $\beta 6$ loop, creates together with L158 a hydrophobic patch that may aid in the release of imipenem by helping rotate the R1 group, and allowing the attacking water to approach the scissile bond (Docquier et al, 2009). The conformation of the $\beta 5$ - $\beta 6$ loop may also influence the conformation adopted by L158 (Smith et al, 2013), which can partially occupy the pocket where the attacking water needs to be placed in, thus competing with it. The $\beta 5$ - $\beta 6$ loop and the Ω -loop may also influence the formation of a network of water molecules, probably having an impact on both the rate at which a water molecule may enter the active site, and its most stable position once inside. The MIC for imipenem for the R214A mutant suggests that maintaining the R214-D219 salt bridge is important for the hydrolysis of imipenem. This interaction might be required to keep the $\beta 5$ - $\beta 6$ loop in the best conformation to facilitate imipenem hydrolysis, or to keep R214 and both loops in a conformation that maintains a good water molecules network. Indeed, the more linear and gradual decrease in turnover rate for imipenem observed for the C-terminus deletion mutants, the first three of which maintain the R214-D159 salt bridge, seems to correlate well with the lesser extent to which these deletions disrupt the conformation adopted by the N-terminus of the loop, compared to the N-terminus deletions. On the other hand, the seemingly higher effect the N-terminus deletions seem to have on the oxyanion hole may also be affecting the turnover rate, and the higher conformational changes on the N-terminus of the

loop may also influence the water molecules network around the active site cavity.

In summary, with our results we propose that the loss of carbapenemase activity is not a problem of affinity but a modification in the turnover number, the differences being stronger when the deletions start in Y211. At the same time, we propose that the reduction in the $\beta 5$ – $\beta 6$ loop, as happen in OXA-163, enlarge the active site cavity leading to the accommodation of ceftazidime reflected in the decrease of the K_m values, the mutants starting from the P217 showing the most significant differences.

Materials and methods

Bacterial strains

K. pneumoniae 11978 was used as a reference strain for OXA-48 cloning experiments (Poirel et al, 2004). *E. coli* TOP10 (Invitrogen, Saint-Aubin, France) and *E. coli* BL21 (DE3) (Novagen, VWR International, Fontenay-sous-Bois, France) were used for cloning experiments.

Antimicrobial agents, susceptibility testing and microbiological techniques

Minimal inhibitory concentration (MIC) values were determined using the Etest technique (BioMérieux, Paris, France) and interpreted according to the EUCAST breakpoints, updated in 2018 (<http://www.eucast.org>).

PCR, cloning experiments, and DNA sequencing

Whole-cell DNA of *K. pneumoniae* 11978 isolate was extracted using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France) and were used as template for PCR using the following primers: preOXA-48A (5'-TATATTGCATTAAGCAAGGG-3'), cloningOXA-48B (5'-AAAAGGATCCCTAGGGAATAATTTTCTGTTTGAGCA-3'). The amplicon obtained was then cloned into the pCR®-Blunt II-TOPO® plasmid (Invitrogen, Illkirch, France) downstream from the pLac promoter and in the same orientation. Specific primers were designed for the different mutations using the program QuikChange Primer Design (Agilent Technologies). pCR®-Blunt II-TOPO® (Invitrogen), harboring the *bla*_{OXA48} gene, was used as a template for the mutagenesis reaction. QuikChange II Site-Directed Mutagenesis Kit (Agilent technologies) was used, following the manufacturer's recommendations, in order to produce two kind of mutations: (i) substitution of each amino acid of $\beta 5$ – $\beta 6$ loop by alanine, and (ii) consecutive deletions in the amino

acids that formed the $\beta 5$ – $\beta 6$ loop, starting, in one case, from the Y211 and continuing in the right direction until the end of the $\beta 5$ – $\beta 6$ loop, and in the other case, starting from the P217 and continuing to the left until the end of the $\beta 5$ – $\beta 6$ loop. Mutagenesis reaction products were transformed in *E. coli* TOP10 (Invitrogen, Saint-Aubin, France). The recombinant pTOPO-OXA-48MUT1-20 plasmids were electroporated into the *E. coli* TOP10 strain; the electroporants were plated on TSA plate containing kanamycin (50 μ g/ml).

The mutated *bla*_{OXA-48-MUT1-20} alleles fragments corresponding to the mature β -lactamase were cloned into the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois, France) using the PCR generated fragment with primers INF-OXA-48Fw (5'-AAGGAGAT ATACATATGGTAGCAAAGGAATGGCAAG-3'), INF-OXA-48Rv (5'-GGTGGTGGTGCTCGAAGGGAATAATTTTTTCTGTTTGAG-3') and the NEBuilder® HiFiDNA Assembly Cloning Kit (New England BioLabs®Inc, United Kingdom), following the manufacturer's instructions. Recombinant plasmids, pET41-OXA-48-MUT1-20, were transformed into chemocompetents *E. coli* strain BL21 (DE3).

Recombinant plasmids were extracted using the Qiagen miniprep kit and both strands of the inserts were sequenced using M13 primers, for the pCR®-Blunt II-TOPO® plasmid (Invitrogen, Illkirch, France), and T7 primers, for pET41b(+) (Novagen, VWR International, Fontenay-sous-Bois, France), with an automated sequencer (ABI Prism 3100, Applied Biosystems). The nucleotide sequences were analyzed using software available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

β -Lactamase purification

Overnight cultures of *E. coli* strain BL21 (DE3) harboring the different pET41b-OXA-48-MUT1-20 were used to inoculate 2 L of LB broth containing 50 mg/L kanamycin. Bacteria were cultured at 37°C until reaching an OD of 0.6 at 600 nm. Expression of the different OXA-48-MUT1-20 was induced overnight at 25°C with 0.2 mM IPTG, as previously described (Dabos et al, 2018). Cultures were centrifuged at 6000 g for 15 min and the pellets resuspended with 10 mL of Buffer A (20 mM Phosphate buffer, 175 mM SO₄K₂, 40 mM Imidazol, pH 7.4). Bacterial cells were disrupted by sonication and the bacterial debris were removed by two consecutive centrifugation steps at 10.000 g for 1

h at 4°C and 48.000 g for 1 h at 4°C. OXA enzymes were purified in one step pseudo-affinity chromatography using a NTA-Nickel column (GE Healthcare, Freiburg, Germany) (Dabos et al, 2018). Protein purity was estimated by SDS–PAGE, pure fractions were pooled and dialyzed against 20mM Hepes SO₄K₂ 50 mM buffer (pH 7) and concentrated by using Vivaspin® columns (GE Healthcare, Freiburg, Germany). Protein concentration was determined by Bradford Protein assay (Bio-Rad, Marnes-La-Coquette, France) (Bradford, 1976).

Kinetic studies

Kinetic parameters of purified OXA-48-MUT1-20 enzymes were determined at 30°C in 100 mM sodium phosphate buffer (pH 7). The k_{cat} and K_m values were determined by analyzing hydrolysis of β -lactams under initial-rate conditions with an ULTROSPEC 2000 model UV spectrophotometer (Amersham Pharmacia Biotech) using the Eadie–Hoffstee linearization of the Michaelis–Menten equation, as previously described (Naas et al, 1998). The different β -lactams were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France).

Homology modelling of mutant structures, molecular docking and molecular dynamics simulations

Three-dimensional structures for the OXA-48 β 5- β 6 loop deletion mutants were created by homology modelling with Modeller (Webb et al, 2016). Ligand structures were generated with 3D Structure Generator CORINA Classic version 3.60 (Molecular Networks GmbH, Nuremberg, Germany). Covalent docking experiments on OXA-48 and on deletion mutants structures were performed using the GOLD suite (CCDC) (Verdonk et al, 2003). Analysis of homology models and docking results were performed with UCSF Chimera package (Pettersen et al, 2004). Molecular dynamics simulations of OXA-48 and of deletion mutants were performed with Gromacs version 4.6 (Pronk et al, 2013) using the OPLS-AA force field (Kaminski et al, 2001). OPLS-AA force field parameters for covalently-bound imipenem were built using a modified version of the MOL2FF package developed in our team. Hop software version 0.4.0 alpha 2 (<https://github.com/Becksteinlab/hop>) was used for water molecule dynamics analysis (Beckstein et al, 2009). Images were created with PyMol (Schrödinger, 2015) and the YRB script was used for surface representations (Hagemans et al, 2015).

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Conflict of interest

The authors declare no competing interests.

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Role of the loop β 5- β 6 in the substrate specificity of OXA-48

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Figures S1-S14. Hydrophobic/charged surface representations, and stick representations of the most representative structure extracted from MD simulations of OXA-48 and deletion mutants.

Stick and ribbon representations of imipenem, L158 and the β 5- β 6 loop, showing the different main conformations adopted by the loop during covalent complex MD simulations. For clarity, only α 3 helix, Ω -loop, β -sheet, β 5- β 6 loop, β 7- α 10 loop and α 10 helix are shown as ribbon. Hydrophobic/charged surface representations of these structures show how the surface of the L158-T213 hydrophobic patch is affected by these mutations, as well as how different mutants partially block the active site cavity with a charged residue or make it narrower. Hydrophobic surfaces colored yellow, positive and negative surfaces colored blue and red, respectively.

Figure S1. OXA-48

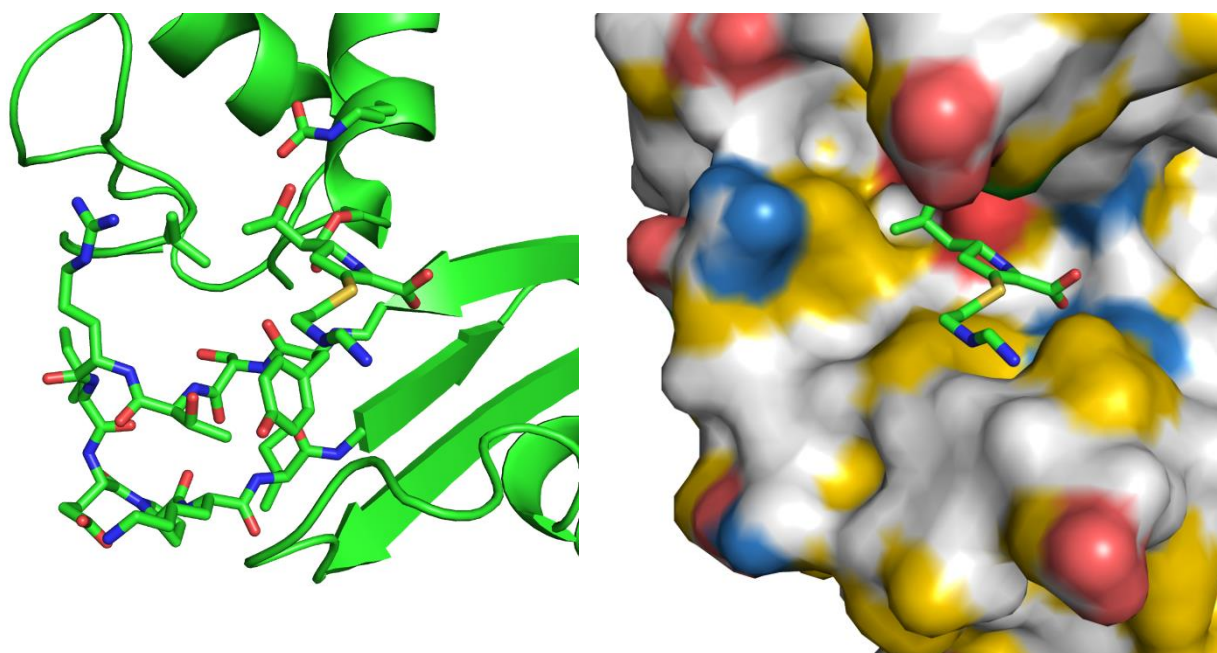


Figure S2. OXA-48 Δ Y

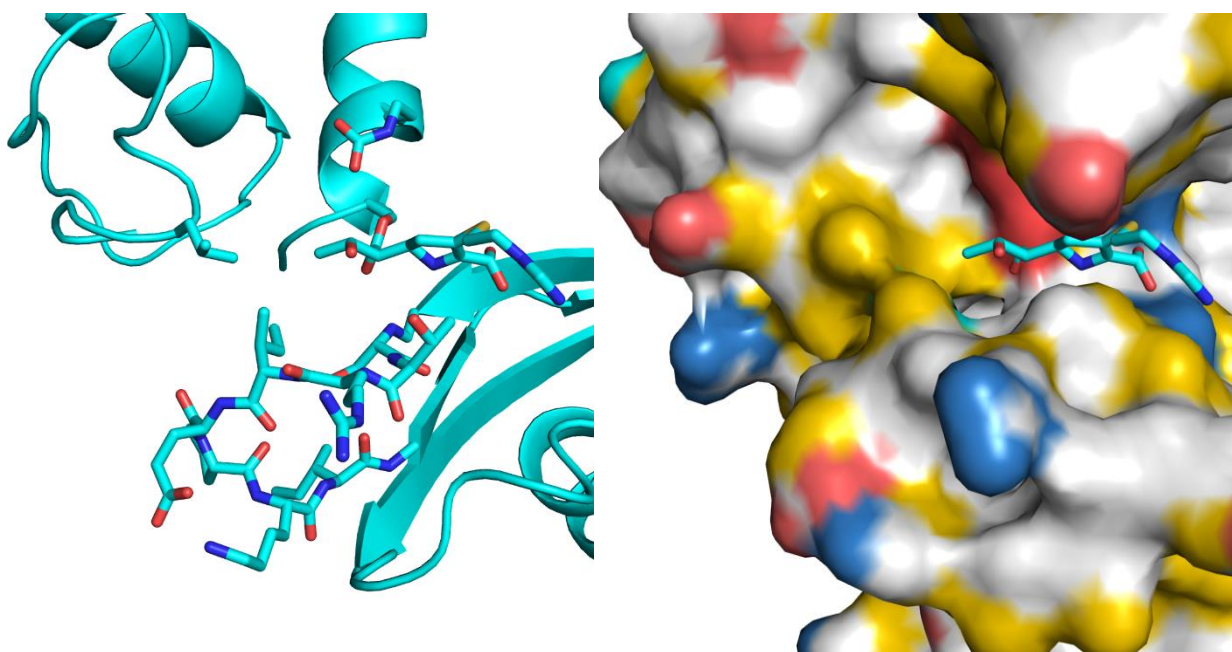


Figure S3. OXA-48 Δ YS

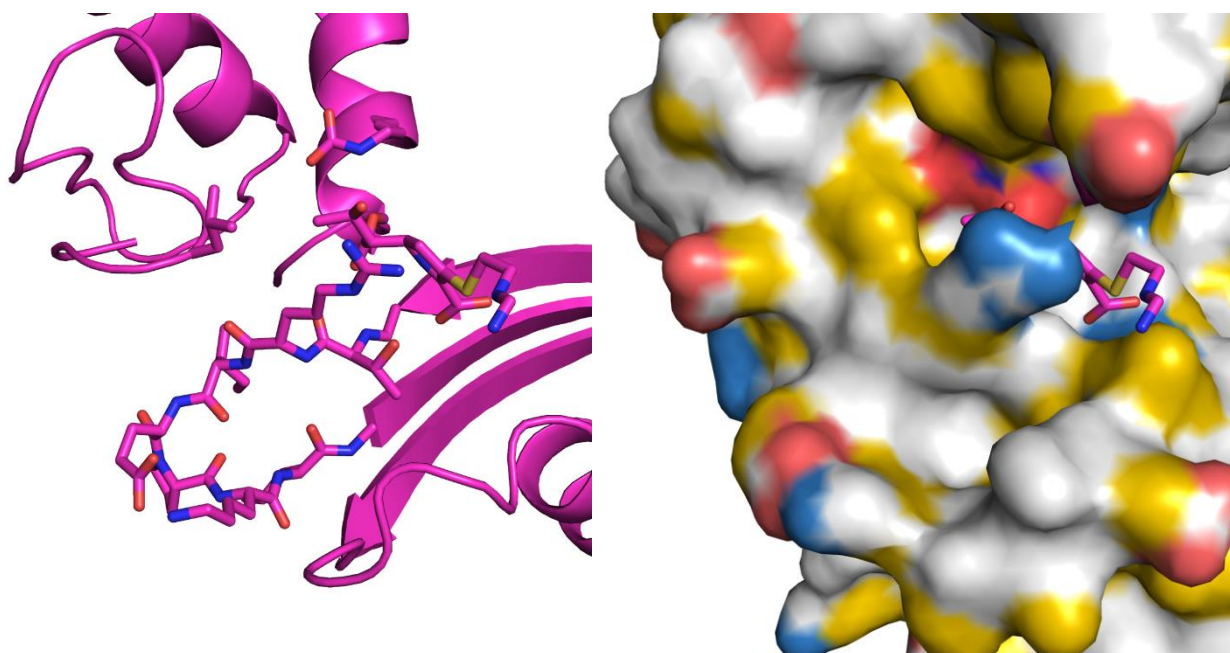


Figure S4. OXA-48 Δ YST

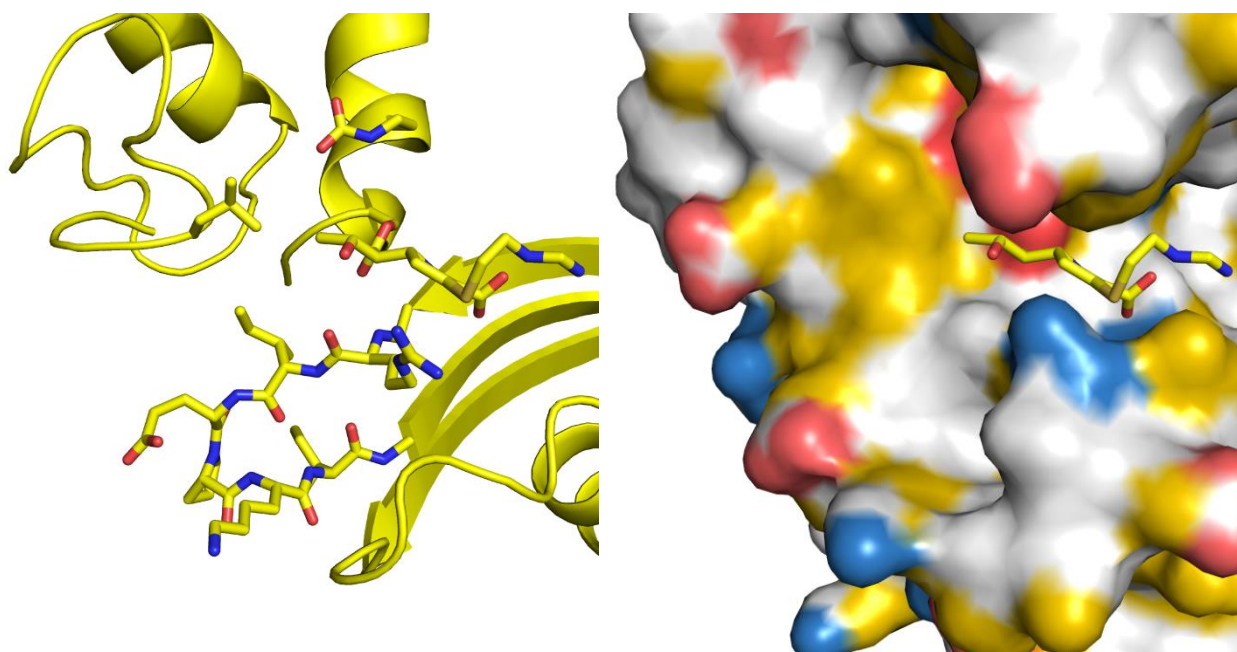


Figure S5. OXA-48 Δ YSTR

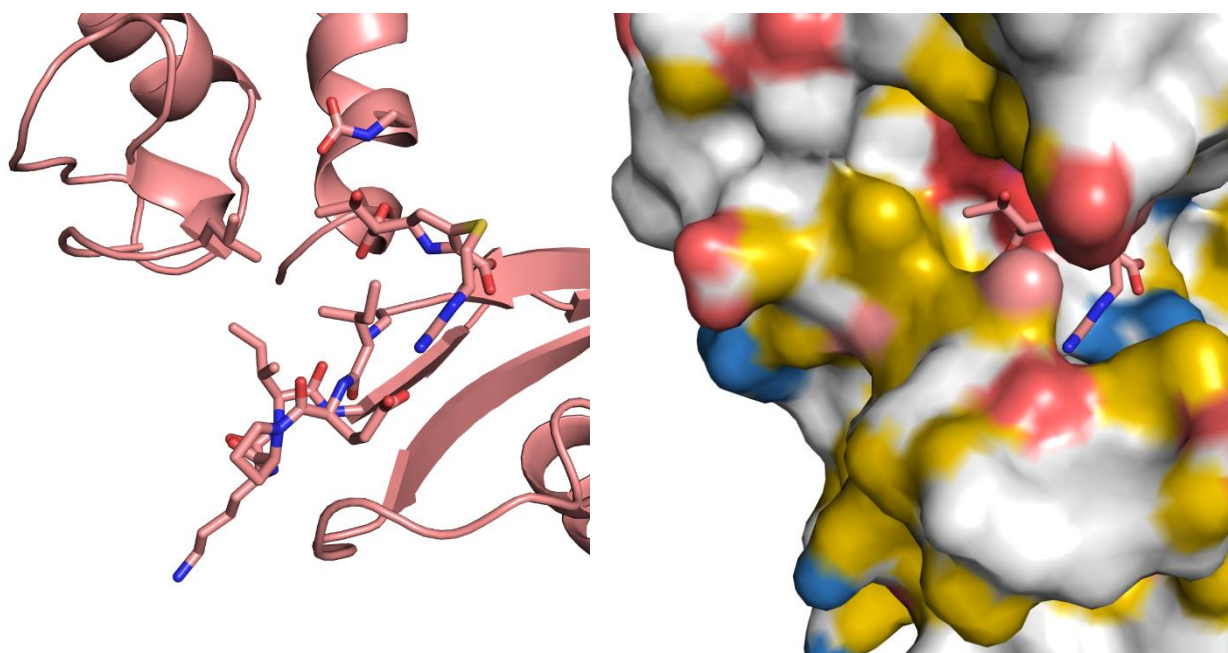


Figure S6. OXA-48 Δ YSTRI

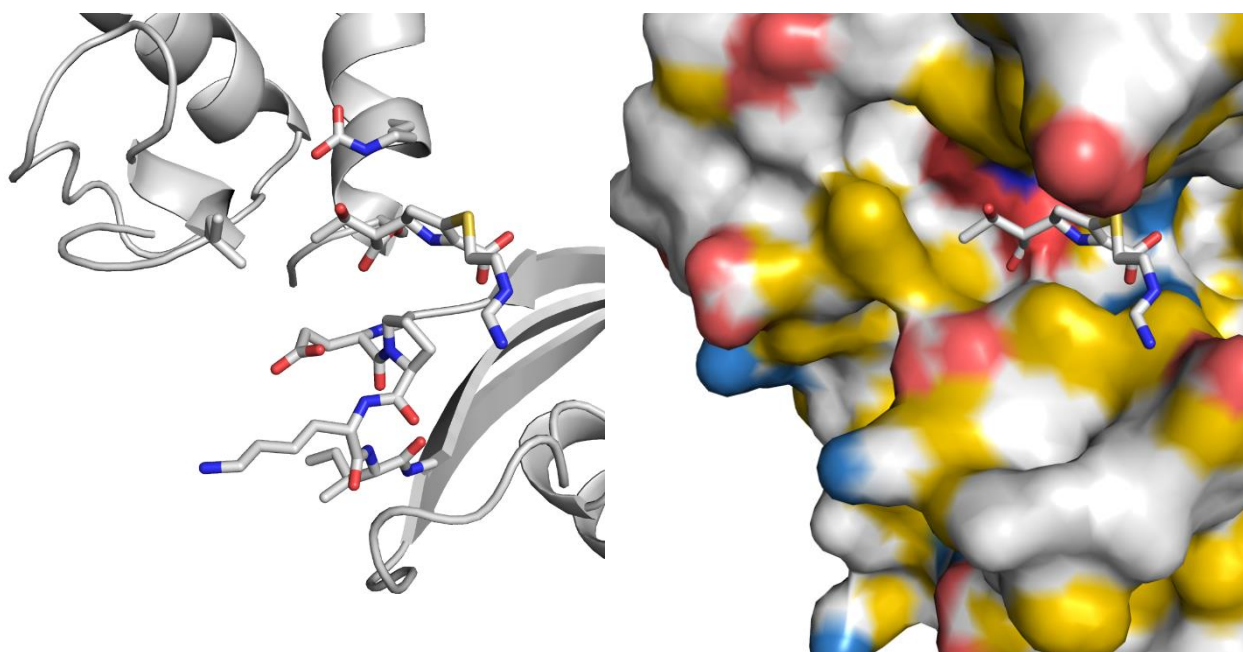


Figure S7. OXA-48 Δ YSTRIE

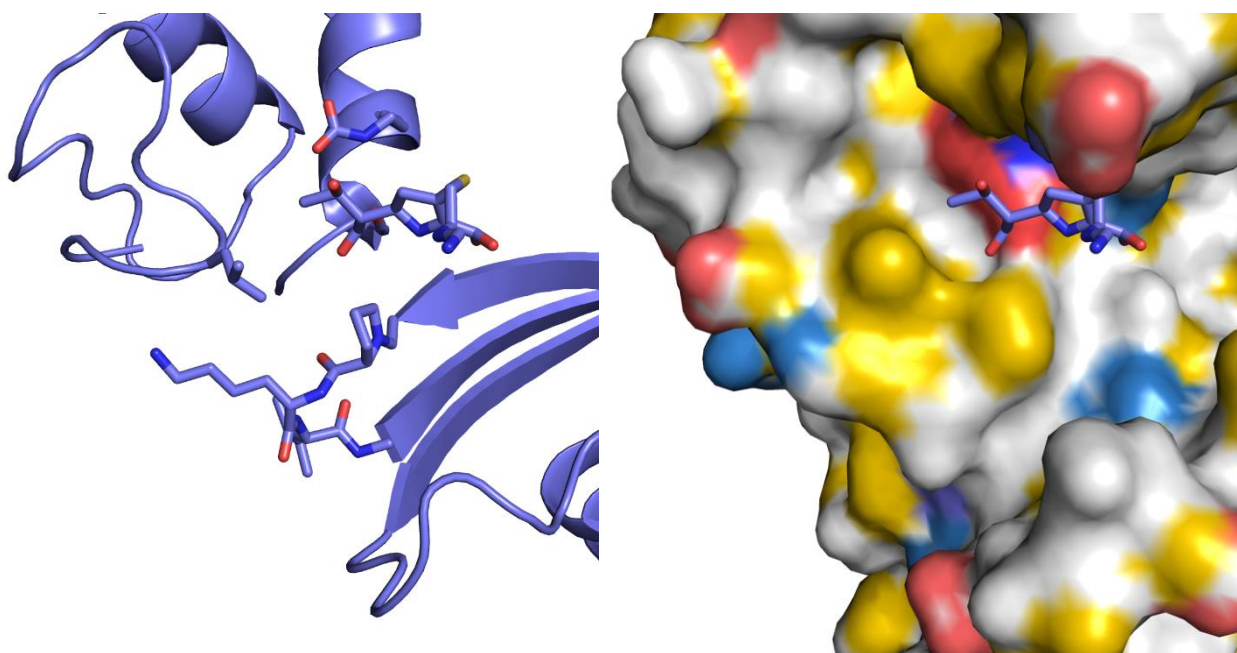


Figure S8. OXA-48 Δ YSTRIEP

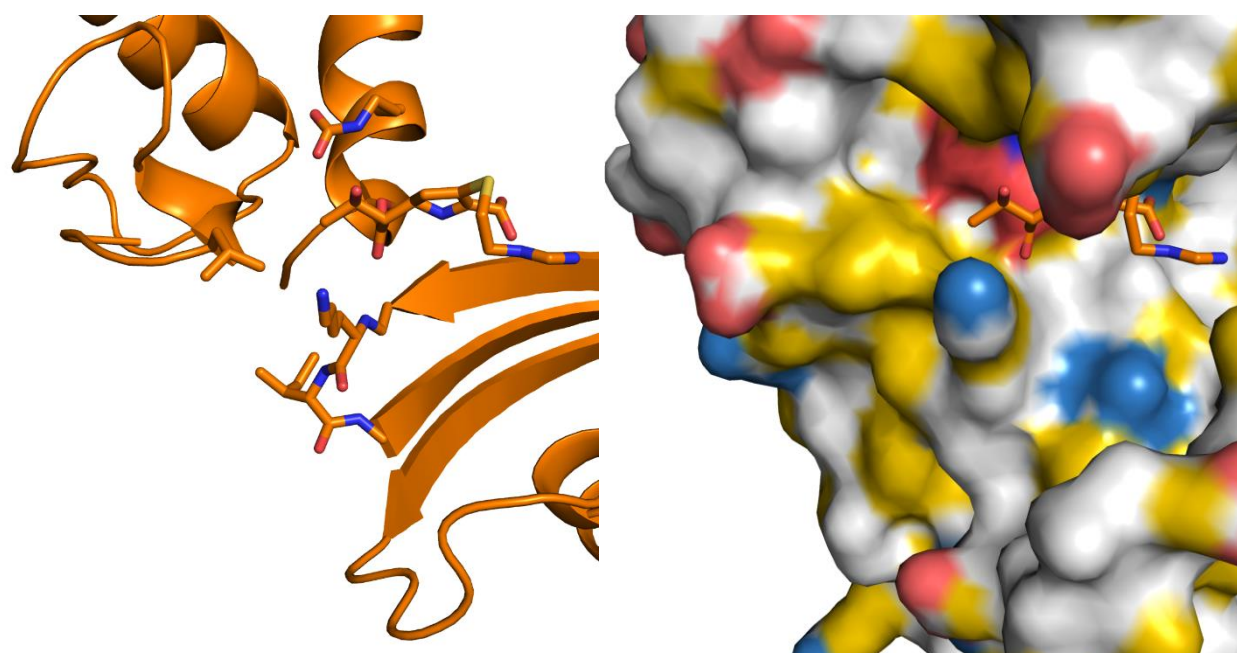


Figure S9. OXA-48 Δ P

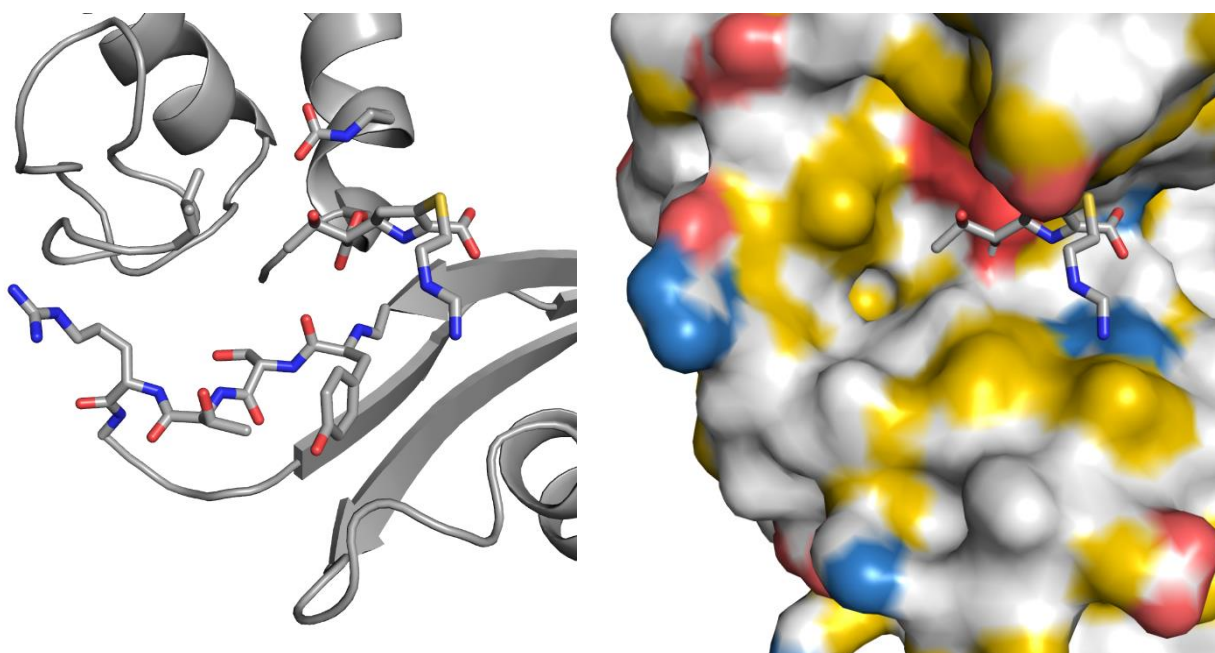


Figure S10. OXA-48 Δ EP

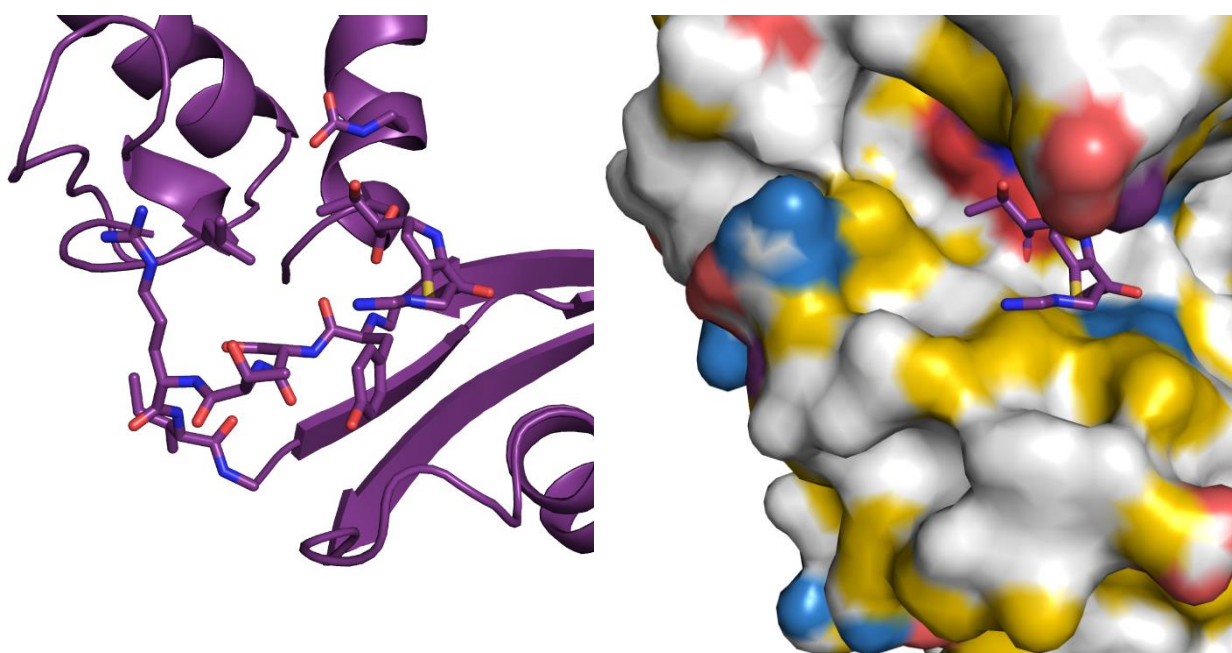


Figure S11. OXA-48 Δ IEP

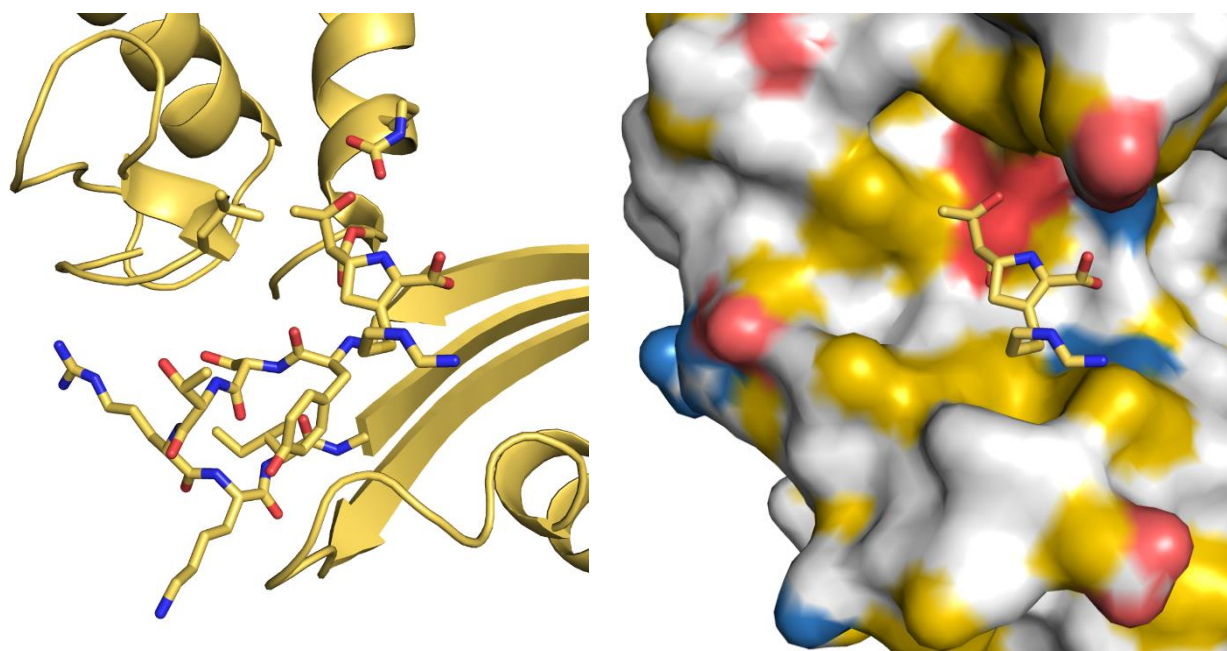


Figure S12. OXA-48 Δ RIEP

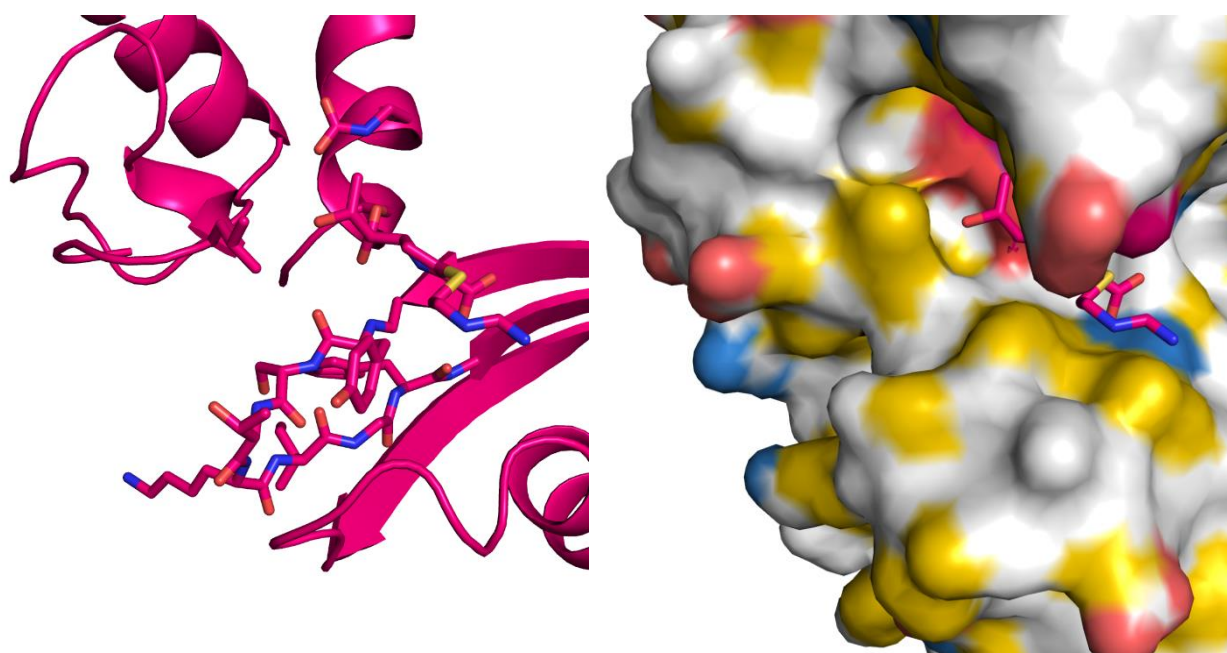


Figure S13. OXA-48 Δ TRIEP

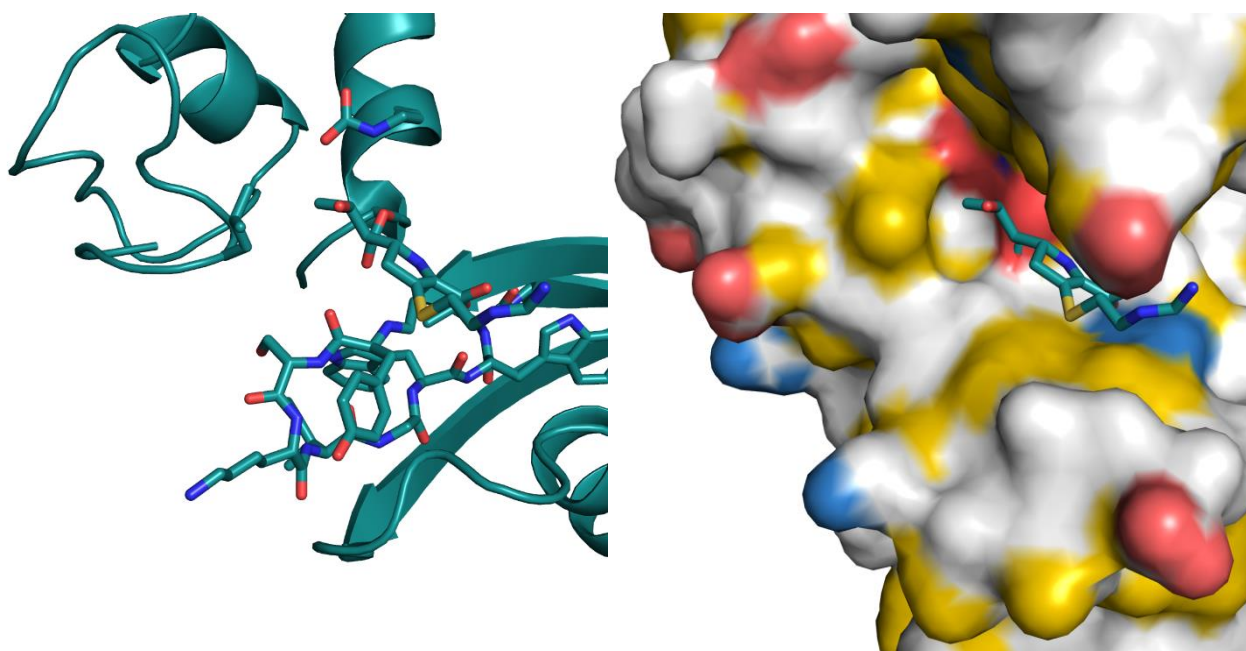
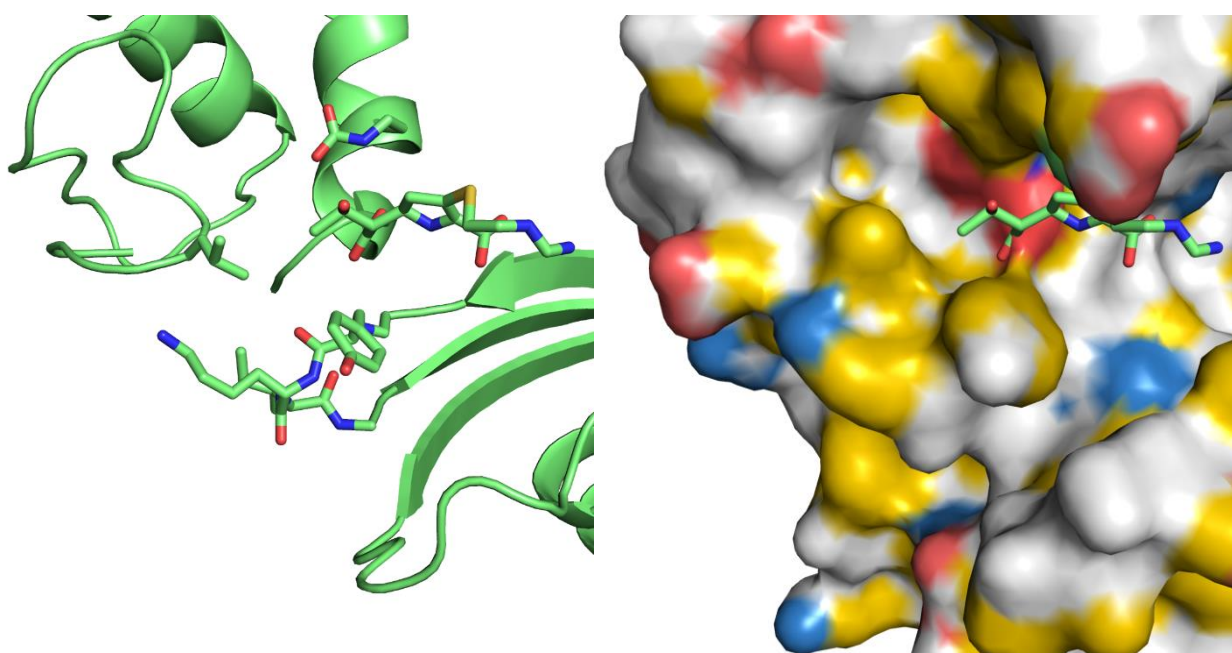


Figure S14. OXA-48 Δ STRIEP



X-ray crystallography of synthetic mutant OXA-48 P217del: nitrate as a class D β -lactamase inhibitor

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Running title: OXA-48 P217del: nitrate as inhibitor of class D β -lactamases

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Keywords: β -lactamase, crystal structure, OXA-48 P217del mutant, inhibitor, nitrate.

ABSTRACT

OXA-48-like carbapenemase producing *Enterobacteriaceae* are now globally disseminated. Among the many naturally occurring OXA-48-like β -lactamases found so far, some variants are reported to present deletions located in the β 5- β 6 loop that result in gain of expanded-spectrum cephalosporin hydrolysis and the loss of carbapenem-hydrolysis. The importance of the β 5- β 6 loop on the OXA-48 hydrolysis profile was investigated by alanine scanning and by tandem deletions of one to six amino acids starting from the N-terminus (Y221) or C-terminus (P217) of the loop. Phenotypical and biochemical analysis of the mutants presented the OXA-48 P217del enzyme as an interesting variant that, unlike the native OXA-48, was capable of hydrolyzing ceftazidime, while keeping the same catalytic efficiency towards imipenem as its parental enzyme. OXA-48 P217del was originally crystallized in order to determine the structural basis of the hydrolytic profile expansion, but it unexpectedly provided information on a different subject. The crystal structure revealed a nitrate anion bound in a subpocket of the active site of OXA-48 P217del, and notable conformational changes such as a displaced and non-carbamoylated K73, a distortion in the β 5 strand and the apparent occlusion of the active site. All these features suggest that the enzyme has adopted a self-inhibited conformation. Inhibition assays confirmed OXA-48 to be inhibited by the nitrate ion, with an IC_{50} comparable to halogens. These results reveal nitrate as a previously unknown class D β -lactamase inhibitor, much in the same manner as halogens, and provides the structural basis for its inhibitory activity.

To be submitted

Introduction

Since their discovery, antibiotics have been a cornerstone of modern medicine, allowing to save countless lives. Antimicrobial resistance (AMR) is one of the biggest threats to global health nowadays, causing 700,000 deaths/year today, but possibly over 10 million deaths/year by 2050 if actions are not taken to tackle it [O'Neill, 2014]. Due to their efficacy and safety, β -lactams are the most utilized antimicrobial therapy, and the most common resistance mechanism is the expression of β -lactamases, which are able to hydrolyze all types of β -lactams, including the latest and most potent ones, carbapenems. β -lactamases can be classified in four classes, A to D [Ambler, 1980]. Classes A, C and D are serine- β -lactamases, presenting an active site serine at position 70, whereas class B β -lactamases are metalloenzymes, with one or two Zn^{2+} ions in the active site [Ambler, 1980]. Class D β -lactamases (DBLs) (also known as oxacillinases or OXA-type β -lactamases) are a very diverse group, with some members sharing only 20% to 30% sequence identity [Naas and Nordmann, 1999]. Most DBLs present either a narrow or extended-spectrum of hydrolysis, and some are capable of hydrolyzing carbapenems [Poirel et al., 2010]. Carbapenem-hydrolyzing class D β -lactamases (CHDLs) were initially found mainly in *Acinetobacter* spp. [Poirel and Nordmann, 2006; Higgins et al., 2009]. OXA-48-like β -lactamases, however, have increasingly been found in *Enterobacteriaceae* isolates [Poirel et al., 2012]. OXA-48 is a carbapenemase that presents high-level of penicillin hydrolysis, and low-level of carbapenem hydrolysis. It hydrolyzes cefotaxime very poorly, and spares completely ceftazidime and cefepime [Docquier et al., 2009; Poirel et al., 2004]. OXA-48 was initially recovered from a *Klebsiella pneumoniae* isolate in 2001 [Poirel et al., 2004]. Since its discovery, almost thirty OXA-48 variants have been reported and several more found in genomic data (<http://blddb.eu/alignment.php?align=D:OXA-48-like>) [Naas et al., 2017; Oueslati et al., 2018]. Most OXA-48-like enzymes present a discrete hydrolytic profile, either including penicillins and carbapenems but sparing expanded-spectrum cephalosporins (e.g. OXA-181 [Potron et al., 2011] and OXA-232 [Potron et al., 2013]), or hydrolyzing penicillins and expanded-spectrum cephalosporins but very weakly (or not at all) carbapenems (e.g. OXA-163 [Poirel et al., 2011] and OXA-405 [Dortet et al., 2015]). Recently, a novel OXA-48-like carbapenemase has been reported, OXA-517, capable of hydrolysing both ceftazidime and imipenem [Dabos et al., 2018b].

Superimposition of DBLs 3D structures reveals they share the same overall fold, with small length and orientation differences mainly in the loops connecting secondary structure elements, such as the β 5- β 6 loop in OXA-48-like enzymes. This loop connects the β 5 and β 6 strands and is located close to the active site groove [Docquier et al., 2009]. The similar size and orientation of the β 5- β 6 loop in CHDLs OXA-24 and OXA-48 suggested a possible involvement of this loop in the ability to hydrolyze carbapenems. Moreover, several non-carbapenemase OXA-48 variants capable of hydrolyzing expanded-spectrum cephalosporins present mutations in the region of the β 5- β 6 loop [Stojanoski et al., 2015; Dortet et al., 2015; Dabos et al., 2018b]. Therefore, research has been carried out exploring this aspect by studying synthetic variants [De Luca et al., 2011; Dabos et al., 2018d, 2018c].

Tandem deletions on the β 5- β 6 loop resulted in the gradual acquisition of cephalosporinase activity and decrease of carbapenemase activity, up to a certain degree depending on the length of the deletion and the starting point (Y211 or P217) [Dabos et al., 2018d]. Together with studies on other naturally occurring mutants [Mitchell et al., 2015;

Potron et al., 2013], results corroborate the relevance of this loop on the substrate profile of OXA-type enzymes, highlight the plasticity of this loop in the evolution of substrate profile under antimicrobial therapy, and point at certain key amino acids such as Y211, R214 and P217 as key residues affecting the impact of the $\beta 5$ - $\beta 6$ loop on the hydrolytic profile of OXA-48 [Dabos et al., 2018d]. OXA-48 P217del turned out to be a very interesting variant that, unlike the native OXA-48, was capable of hydrolysing ceftazidime, while keeping the same catalytic efficiency towards imipenem as the parental enzyme (Table 1). In order to investigate the structural basis of the hydrolytic profile expansion in OXA-48 P217del, the enzyme was crystalized and its X-ray structure determined. Analysis of OXA-48 P217del crystal structure reveals a non-native structure with a distorted conformation compared to almost all other class D structures. Comparison to the structure of OXA-163 complexed with an iodide anion suggests that the presence of a nitrate ion in the OXA-48 P217del structure might drive the adoption of this conformation.

Table 1: Kinetic parameters of OXA-48 and OXA-48 P217del

β -lactam	K_m (μ M)		k_{cat} (s^{-1})		k_{cat}/K_m (mM^{-1}/s^{-1})	
	OXA-48	OXA-48 P217del	OXA-48	OXA-48 P217del	OXA-48	OXA-48 P217del
Ampicillin	395	264	955	99	2388	374
Aztreonam	NH	>1000	NH	>0.35	NH	0.32
Cefalotin	195	175	44	6.8	226	39
Ceftazidime	NH	42	NH	0.05	NH	1.2
Imipenem	13	8	4.8	3	369	386
Temocillin	45	530	0.3	3.1	6.7	5.9

Taken from [Dabos et al., 2018d]

Results and discussion

OXA-48 P217del crystallization and X-ray crystallography:

OXA-48 P217del crystallized in a solution containing 0.2 M sodium nitrate and 20% w/v PEG 3350. The structure was determined by molecular replacement using the deposited OXA-48 structure (PDB: 3HBR) as a template, and the model was refined to 1.75 Å (Table 2). The asymmetric unit contains two protein chains in space group $P2_12_12_1$, modelled with 244 residues for chain A and 243 for chain B. Both chains present the typical class D fold with an α -helical region and a mixed α -helix/ β -sheet region, with a C α RMSD of 0.62 Å compared to the OXA-48 structure. Detailed electron density is observed for backbone and sidechains for both protein chains (Fig. 1), allowing over 95% of the residues to be inside the favoured regions of the Ramachandran plot, with the exception of the loop $\beta 7$ - $\alpha 10$ on chain B, for which the trace is fading away for P241-S243. Backbone B-factors indicate this loop to be the most flexible portion of the crystal structure, on both chains, together with the C- and N- termini, followed by the N-terminus of $\alpha 1$ helix, and the $\beta 5$ - $\beta 6$ loop, both structures adjacent to the $\beta 7$ - $\alpha 10$ loop. The $\alpha 3$ - $\alpha 4$ loop also has relatively high B-factor values, mainly

on chain A. Several ordered water molecules have been modelled in the structure as well as other components from the protein or crystallization solution or the cryoprotectant (i.e

Table 2: X-ray crystallography and refinement statistics

Data collection	
Space group	P 2 ₁ 2 ₁ 2 ₁
Cell dimensions	
a, b, c (Å)	49.57, 92.98, 126.39
α , β , γ (°)	90.00, 90.00, 90.00
Resolution (Å)	25.61-1.75
R _{meas}	5.60%
I/ σ (I)	1.97 (at 1.75Å)
Completeness (%)	97.5
Redundancy	3.9
Refinement	
Resolution range (Å)	25.61-1.75
No. unique reflections	58,856
R _{work} /R _{free}	17.0%/18.5%
No. non-hydrogen atoms	
Protein	4,125
Water	376
Ligand/Ions	159
Total	4,660
Average B, all atoms (Å ²)	33.34
Protein	32.34
Water	47.30
Ligand/Ions	51.84
Root mean squared deviations	
Bond lengths (Å)	0.01
Bond angles (°)	0.96

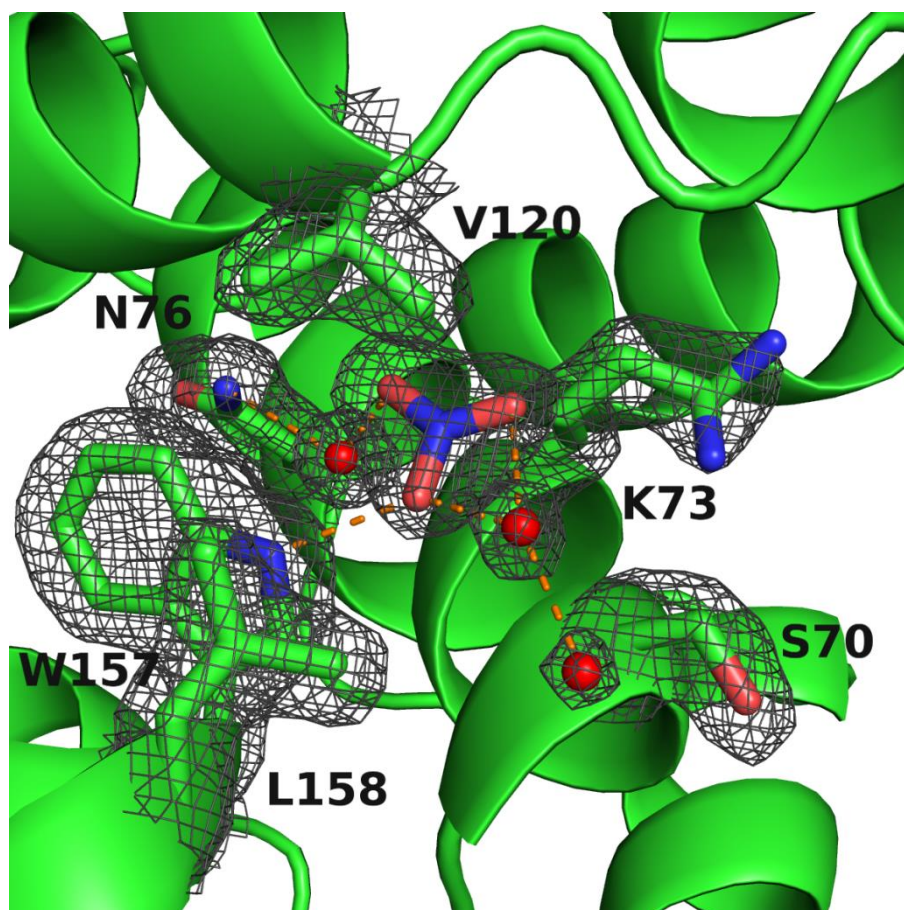


Figure 1. Crystal structure of OXA-48 P217del. Clear electron density is observed for active site cavity residues and waters of chain A, as well as for the nitrate anion bound next to, and displacing, K73. Hydrogen bonds are depicted as dashed orange lines, 2Fo-Fc map is contoured at 1.0 σ level.

phosphate, nitrate, ethanediol, PEG and glycerol). No significant differences can be observed between both chains, except for a slight shift in the space occupied by the flexible $\beta 7$ - $\alpha 10$ loop, modelled closer to the $\alpha 3$ - $\alpha 4$ loop in chain B. Chains A and B form the dimeric structure already described for OXA-48 [Docquier et al., 2009] and a chloride anion is observed at the dimeric interface, bridging between the R206 residue of both chains.

The structure of OXA-48 P217del reveals a number of important differences compared to the structure of native OXA-48, showing a distorted conformation of the active site cavity (Fig. 2). The conformation of certain residues surrounding the cavity is conserved, such as A69 and N76 on the $\alpha 3$ helix, S118 and V120 on the $\alpha 4$ - $\alpha 5$ loop, Y123 and N124 on the $\alpha 5$ helix, and W157 and L158 on the Ω loop. A nitrate anion can be observed buried in the active site cavity surrounded by K73, V120, Y123, W157, L158, S70 and A69. It occupies the same space the planar functional group of the carbamoylated lysine does in the OXA-48 structure, making hydrogen bonds to W157 and N76 via a water molecule, just like KCX in OXA-48. Unlike KCX, nitrate in OXA-48 P217del does not interact with S70, which is displaced towards the $\beta 6$ strand and the C β -O γ bond is rotated 120°, now interacting with a water molecule located between the $\beta 5$ and $\beta 6$ strands and the backbone

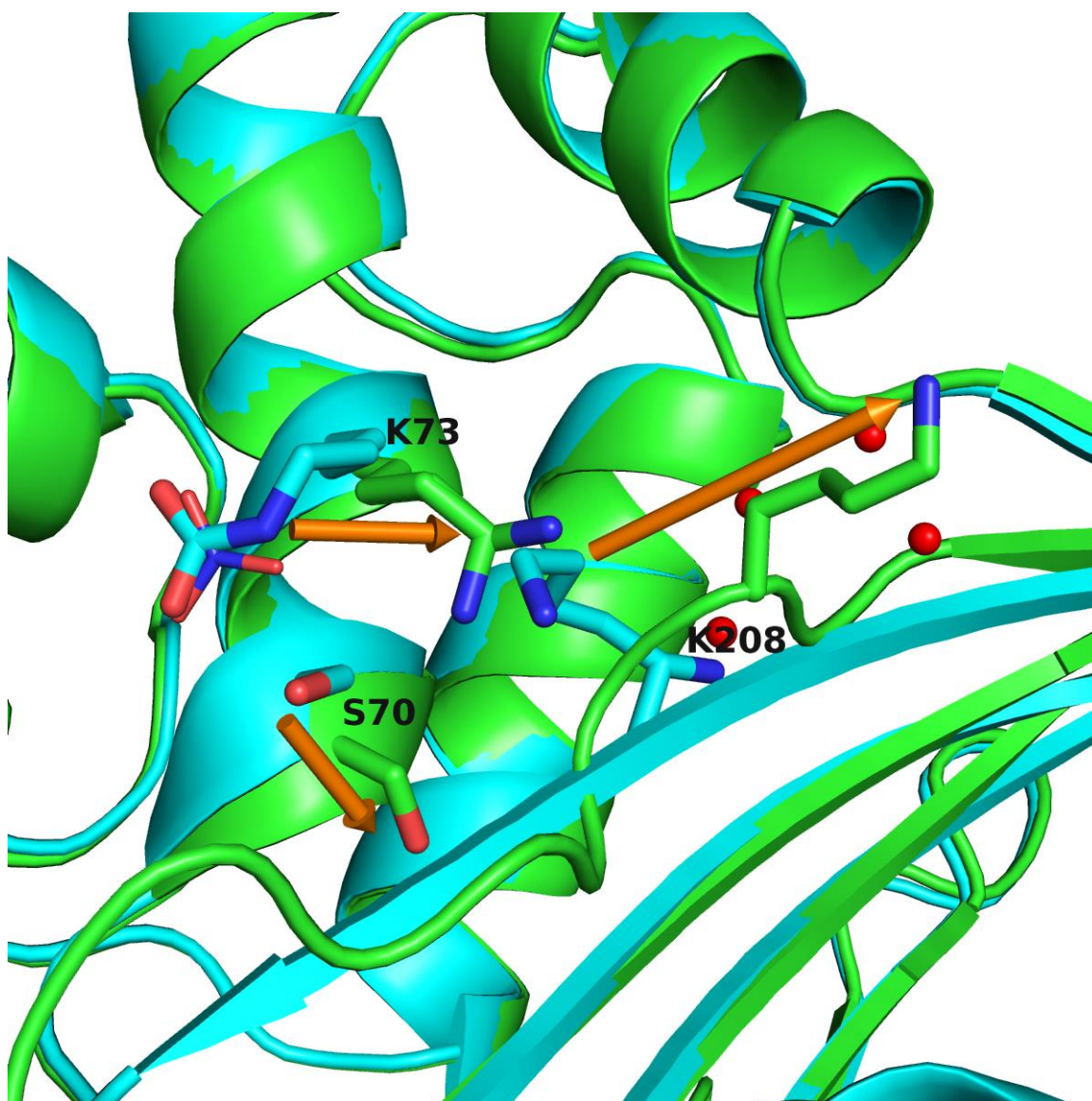


Figure 2. Displacement of active site cavity residues by nitrate. Superposition of OXA-48 structure (coloured in cyan, PDB 3HBR) and the OXA-48 P217del structure (coloured in green) shows the conformational change caused by the nitrate anion on K73, S70 and K208. Notice the approximately 120° rotation of S70 and the ordered water molecules displaced by the displacement of K208.

oxygen of T209. Instead, a water molecule located close to the native OXA-48 S70 O_{γ} position makes hydrogen bonds with the nitrate anion and is also stabilized by interactions with other water molecules in the active site cavity or with T213 (this latter interaction is observed only in chain A). K73 is shifted towards K208 from the KTG motif, causing a 5 Å displacement of its N_{ζ} (Fig. 2). The K208 sidechain is also displaced from its native conformation by K73, towards the β_4 strand, displacing several water molecules and making a hydrogen bond with the backbone oxygen of the dimer interface residue L196. The conformational changes also extend to the β_5 - β_6 loop and to the strands connected by this loop. Starting from K208, the β_5 strand twists and detaches from the β -sheet (Fig. 2), losing 6 hydrogen bonds connecting it to the β_6 strand.

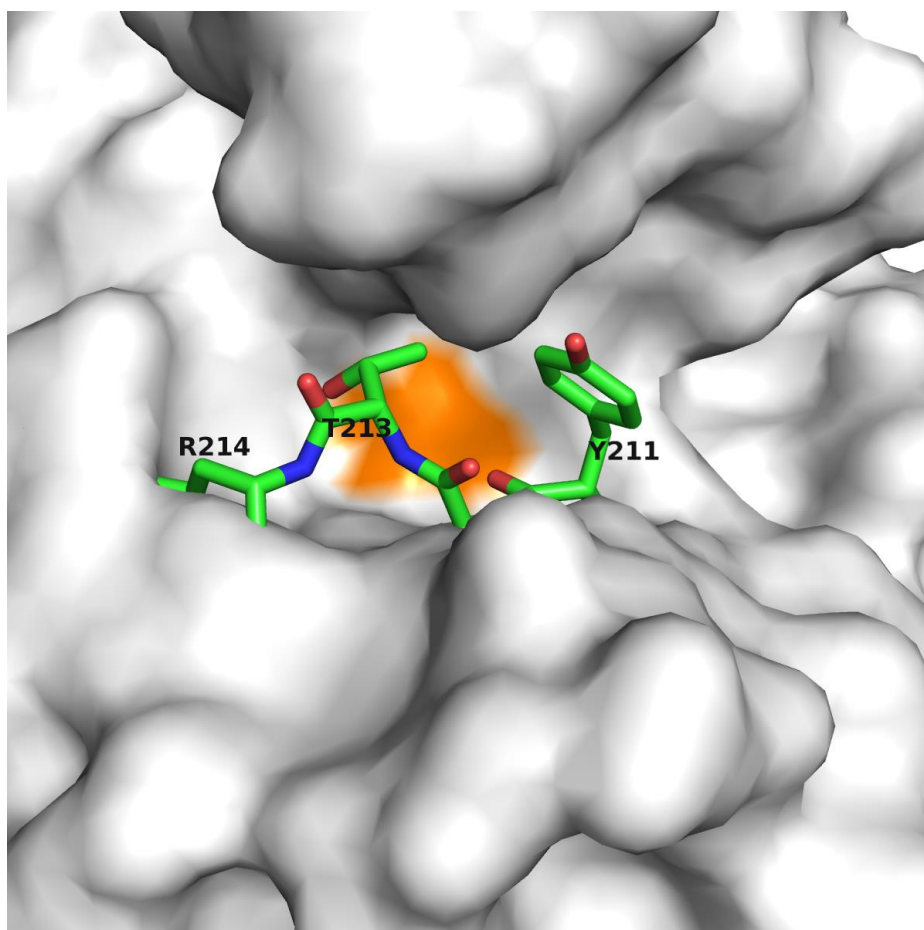


Figure 3. Occlusion of the active site cavity in OXA-48 P217del. Superposition of OXA-48 P217del crystal structure (green sticks) on the OXA-48 structure (white surface, PDB 3HBR) shows how the conformational change of strand $\beta 5$ and loop $\beta 5$ - $\beta 6$ makes residues 211-YSTR-214 occlude the active site cavity in the OXA-48 P217del structure, hiding the active site S70 (orange surface).

Because of the twist, the sidechains now point in the opposite direction they usually do, aiding K208 to interact with L196, and making T209 to point inwards. As the $\beta 5$ detaches, the $\beta 5$ - $\beta 6$ loop residues Y211-T213 occupy the space in front of the active site floor, effectively occluding the active site serine (Fig. 3). A few water molecules can be observed in the crystal structure occupying the wider space left between both strands towards the $\beta 5$ - $\beta 6$ loop. Curiously, the salt bridge between R214 and D159 is maintained in spite of the almost 6 Å displacement of the corresponding Ca . D216 and K218 (OXA-48 numbering is kept throughout the text for convenience, disregarding the -1 shift caused by the P217 deletion) are adjacent to one another in this structure, and a salt bridge between their sidechains is not observed here. The backbone of the $\beta 6$ strand is somewhat displaced from its original conformation, up to W222. A change in the conformation of L247 and R250 can also be seen, with R250 buried deeper into the enzyme pushed by the backbone of Y211 in its new conformation, and L247 displaced towards Q251, where it would clash with R250 and T209 if they weren't displaced in this structure. The conformation of these last two residues may also be influenced by the interaction of the chain B $\alpha 3$ - $\alpha 4$ loop from an adjacent unit cell.

We report here the first crystal structure class D β -lactamases that contains a nitrate anion. The structures of three other β -lactamases (belonging to classes A and C) containing a nitrate anion are present in the PDB (PDB codes 5U53, 5E43, 4NET [Patel et al., 2017; Bhattacharya et al., 2014]), but in these cases it seems that the nitrate has no significant influence on the overall conformation of the enzyme.

The conformational changes observed in this OXA-48 P217del structure show several points in common with the ones observed in the structure of OXA-163 in complex with iodide (PDB code 4S2M) [Stojanoski et al., 2015]. Nitrate is observed occupying the same position as iodide, and the displacement of K73 and K208 is very similar in the two structures (Fig. 4). S70 is also turned around the C β -O γ bond in the same manner in the OXA-163 structure, and a detachment of the β 5 strand and occlusion of the active site serine is also observed, although in the OXA-48 P217del structure it seems to reach further towards the α 3- α 4 loop, possibly because its β 5- β 6 loop (one deletion) is longer than that of OXA-163 (four deletions). There is also a slight displacement of R250 in OXA-163, but more restricted than in OXA-48 P217del, and the conformation of the β 7- α 10 loop in OXA-163 is not well defined either in any of its chains.

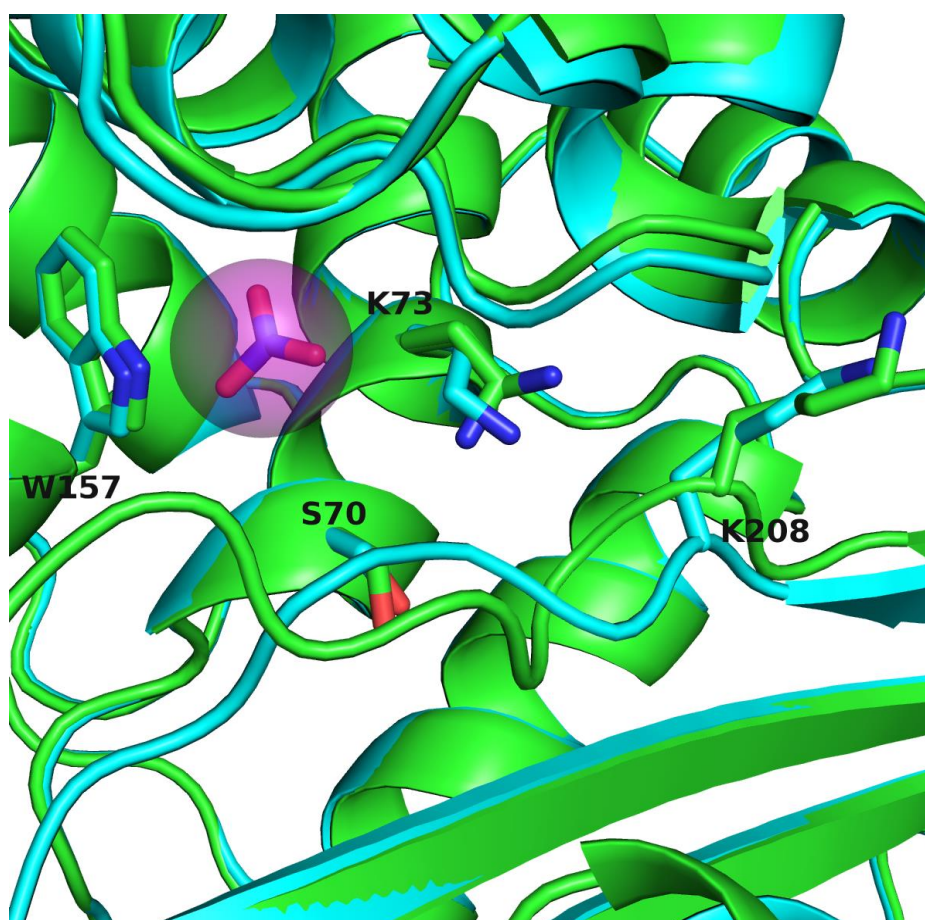


Figure 4. Similar conformational changes in the iodine/OXA-163 and nitrate/OXA-48 P217del complexes. Superposition of the iodine/OXA-163 (transparent purple surface and cyan sticks, respectively) and nitrate/OXA-48 P217del (blue and green sticks, respectively) complexes evidences similar conformational changes caused by both anions on the protein structures. Notice the similarities in the space iodide and nitrate occupy, the displacement of K73, S70, K208 and the detachment of strand β 5 from the β -sheet, causing the occlusion of the active site cavities of both enzymes.

The fact that the displacement of K73 has been observed with nitrate supports the idea proposed by Stojanoski *et al.* [Stojanoski et al., 2015] that although other halogens such as chloride may bind in the same position and inhibit class D enzymes, as seen for OXA-10 (PDB 2WGV) [Vercheval et al., 2010], only bigger anions (such as iodide or nitrate, and probably bromide as well) may cause this conformational change. Structures of OXA-10 with iodide and bromide have been reported (PDB codes 5MOZ, 5MNU [Lohans et al., 2017]), where these conformational change is not observed. There are differences, however, in the residues surrounding K73 in these two enzymes (e.g. Y123 in OXA-48 is F120 in OXA-10), and the energetic cost of the conformational change implied for OXA-10 might be higher due to these differences in amino acid sequence. Moreover, the conformational change of K208 in OXA-48 would displace a cluster of water molecules, whereas the same displacement (K205 in OXA-10) would significantly overlap with (and thus require the displacement of) the Q113 sidechain, which is interacting with the extended β 4 sheet taking part in the dimer interface of OXA-10. Q113 would present severe clashes with its own polypeptide or the extended β -sheet coming from the other monomer if it were to change conformations. In OXA-48, the analogous residue is K116, which is also participating in the dimer interface, but pointing in a different direction and making different interactions, which are not disturbed by the displacement of K208. This would imply a higher energetic cost for the cascade of sidechain displacement events in OXA-10 than in OXA-48, perhaps even disrupting the dimeric structure.

It has been reported that class D enzymes presenting Phe instead of Tyr at the YGN motif are resistant to inhibition by chloride [Héritier et al., 2003]. Although this residue is located far from the active site cavity (as noted by Stojanoski *et al.* [Stojanoski et al., 2015]), its sidechain hydroxyl interacts via a buried water molecule with the backbone oxygen of T71 (part of the SXXK motif on the α 3 helix) and the sidechain of Q169 via a buried water molecule, which also makes a hydrogen bond with the sidechain of T71 and is in the proximity of W220 on the β 6 strand. Thus, mutations on this residue may alter the activity or inhibitor susceptibility of oxacillinases via these interactions, although how this influence may be realized is not obvious from the observation of the crystal structures, and may be a matter of energetics or flexibility. This water molecule is very conserved and present in almost all structures of YGN-containing class D β -lactamases deposited in the PDB. In the structures of oxacillinases with the FGN motif (i.e. OXA-23, OXA-24/40, OXA-143, OXA-160, OXA-225, OXA-239), conversely, only one of them has been modelled with a water molecule close to the same position, but at 0.5 occupancy, water 656 in OXA-146 (PDB code 4K0W [Kaitany et al., 2013]). Curiously, this OXA-146 structure is not carbamoylated, which was already reported to occur under acidic pH conditions, but also presents a sodium cation in a very similar position to iodide and nitrate in the OXA-163 and OXA-48 P217del structures, respectively. This water molecule connecting the YGN motif to the α 3 helix should also be very stable, as suggested by molecular dynamics (MD) simulations (A. Zavala, B.I. Iorga, personal communication) that show that when not included from the beginning, this cavity is not filled with a water molecule during a 10 ns simulation, whereas, when included from the beginning, the water molecule is never exchanged with the bulk solvent molecules. As a side note, the MD simulations also show that this water molecule may also interact with the sidechain of T71.

Nitrate as inhibitor of OXA-48:

Given that halogens are known to be inhibitors of class D β -lactamases [Naas and Nordmann, 1999; Stojanoski et al., 2015], and as the conformational changes caused by nitrate in the crystal structure resemble those observed in the OXA-163 structure with iodide, we decided to look into

the possibility that nitrate may inhibit oxacillinases as well. IC₅₀ was determined for OXA-48 for nitrate and compared with known values for halogenides (Table 3). Our inhibition results on OXA-48 show that nitrate is slightly more potent than chloride, but less so than iodide or bromide.

Table 3: IC₅₀ values for OXA-48 with halogenides and nitrate anions

Inhibitor	IC ₅₀ OXA-48 (mM)
NaNO ₃ ^a	100 ± 10
NaF ^b	207 ± 11
NaCl ^b	109 ± 12
NaBr ^b	35 ± 7
NaI ^b	14 ± 5

^athis work

^bfrom [Stojanoski et al., 2015]

Conclusion

In this study we report the crystal structure of the 217ΔP synthetic mutant of the OXA-48 β-lactamase. This structure contains a nitrate anion bound in a subpocket of the active site, which induces notable conformational changes such as a displaced and non-carbamoylated K73, a distortion in the β5 strand and the apparent occlusion of the active site, suggesting that the enzyme has adopted a self-inhibited conformation. Inhibition assays confirmed OXA-48 to be inhibited by the nitrate ion, with an IC₅₀ value comparable to chloride. These results reveal nitrate as a previously unknown class D β-lactamase inhibitor and provides the structural basis for its inhibitory activity.

Materials and methods

OXA-48 P217del cloning, over-expression and purification:

OXA-48 P217del was obtained as previously described [Dabos et al., 2018d]. Briefly, *bla*_{OXA-48} was cloned into a pCR®-Blunt II-TOPO® plasmid, and the QuikChange II Site-Directed Mutagenesis Kit (Agilent technologies) was used to create the P217 deletion mutant. *bla*_{OXA-48 P217del} was then subcloned into expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois, France) and inserted into *E. coli* strain BL21 (DE3) for overexpression. Protein was overexpressed overnight in 2 liters of LB broth containing 50 mg/L kanamycin, the cells harvested, and disrupted by sonication. Lysate was clarified and the protein was purified in one step pseudo-affinity chromatography using a NTA-Nickel column (GE Healthcare, Freiburg, Germany) [Dabos et al., 2018a]. Protein purity was estimated by SDS–PAGE, pure fractions were pooled and dialyzed against sodium phosphate 0.1 M, potassium sulfate 50 mM buffer at pH 7.0 and concentrated by using Vivaspin® columns (GE Healthcare, Freiburg, Germany) up to 12.5 mg/ml, and then stored at -70 °C.

Protein crystallization and X-ray crystallography:

Conditions for OXA-48 P217del (12.5 mg/ml) were identified from screening stochastic crystallization conditions among the commercially available suites Classics, AmSO₄, PEGs and PEGs II (Qiagen/NeXtal), using the Mosquito® HTS (TTP LabTech). One single cubic-shaped crystal was obtained in 0.2 M sodium nitrate and 20 % w/v PEG 3350. The crystal was transferred to a cryo-protectant solution consisting of the mother liquor supplemented with 25 % v/v glycerol, then flash-frozen in liquid nitrogen. Diffraction data was collected at 100 K in a nitrogen cryostream on the PROXIMA1 beamline at the SOLEIL synchrotron (Saint-Aubin, France). The data were indexed and integrated with XDS [Kabsch, 2010] via the XDSME script (<https://github.com/legrandp/xdsme> [Kabsch, 2010]). Data scaling was performed using AIMLESS [Evans and Murshudov, 2013] from the CCP4 suite [Winn et al., 2011]. Data collection and refinement statistics are given in Table 2. The structure of OXA-48 P217del with two molecules per asymmetric unit was successfully solved using the automatic molecular replacement program MrBUMP [Keegan et al., 2007] with OXA-48 (PDB entry 3HBR; 99 % sequence identity) as a search model. The model was rebuilt manually in Coot [Emsley et al., 2010] and then refined using BUSTER-TNT [Bricogne et al., 2011] with local noncrystallographic symmetry (NCS) restraints and a translation–libration–screw (TLS) description of B-factors [Murshudov et al., 2011]. The quality of the final refined model was assessed using MolProbity [Chen et al., 2010]. Crystal structure images were generated using PyMOL (<http://www.pymol.org>) [Schrödinger, 2015].

Protein inhibition assays:

IC₅₀ of nitrate and halogens on purified OXA-48 P217del were determined at 30°C in 100 mM sodium phosphate buffer, pH 7.0, by analysing hydrolysis of imipenem under initial-rate conditions with an ULTROSPEC 2000 model UV spectrophotometer (Amersham Pharmacia Biotech) using the Eadie–Hoffstee linearization of the Michaelis–Menten equation, as previously described [Naas et al., 1998]. IC₅₀ was determined following hydrolysis of imipenem 100 µM as a reporter, after preincubation of the enzyme with increasing concentrations of the inhibitors in the presence of NaHCO₃ 50 mM. IC₅₀ was determined as the concentration of inhibitor that reduced the initial hydrolysis rate of the enzyme to 50%, compared to the untreated enzyme. The imipenem and other chemicals were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France).

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Structural and biochemical characterization of OXA-427, a peculiar class D β -lactamase from the OXA-12-like family.

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Running title: *Characterization of OXA-427 β -lactamase*

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Keywords: beta-lactamase, X-ray crystallography, molecular modelling, antibiotic resistance, OXA-427, cephalosporinase, carbapenemase.

ABSTRACT

Antimicrobial resistance is a serious threat to public health, driven by the widespread use of antimicrobial drugs in the past decades. Among antimicrobials, β -lactams are the most used, and the most important resistance mechanism is represented by the expression of β -lactamases. Class D β -lactamases, also known as oxacillinases due to their substrate preference for isoxazolyl-type penicillins, comprise (i) narrow spectrum enzymes like OXA-1 and its variants, hydrolysing mostly penicillins and oxacillin, (ii) extended spectrum β -lactamases (ESBL) like OXA-2 or OXA-10 variants, hydrolysing both penicillins and advanced-generation cephalosporins, but not carbapenems, and (iii) carbapenemases like OXA-48-like, OXA-24/40-like or OXA-51-like enzymes, hydrolysing mostly penicillins and carbapenems but generally not expanded spectrum cephalosporins. Recently, a novel class D carbapenemase showing unusual hydrolytic properties has been reported in Leuven, Belgium, named OXA-427. It confers resistance to broad-spectrum penicillins, extended-spectrum cephalosporins, and carbapenems. Here we report the crystallization and structural characterization of this enzyme, which may provide the structural basis for its unusual resistance profile.

INTRODUCTION

Class D β -lactamases, either chromosomal or acquired, are frequently identified in pathogenic isolates [Poirel et al., 2010][Evans and Amyes, 2014]. For historical reason, most of them are also called OXA because of their relatively strong hydrolytic activity on oxacillin. Class D is composed of diverse families with various hydrolysis profiles toward β -lactams. Several class D extended-spectrum β -lactamases (ESBLs) have been identified (e.g. OXA-11, OXA-14), belonging to OXA-10-like family. Other class D enzymes, termed carbapenem-hydrolysing class D β -lactamases (CHDLs), can hydrolyze carbapenems. CHDLs are usually found in certain bacterial genera, e.g. OXA-48 in *Enterobacteriaceae*, OXA-198 in *P. aeruginosa*, and OXA-23, OXA-24/40, OXA-58 or OXA-143 mostly in *Acinetobacter baumannii* [Evans and Amyes, 2014]. Their catalytic efficiency towards carbapenems is usually low [Docquier and Mangani, 2016], and none of these enzymes possess the ability to hydrolyze significantly both expanded-spectrum cephalosporins and carbapenems. Nevertheless, these enzymes can confer resistance to carbapenems in clinical isolates, usually paired with impermeability or efflux resistance mechanisms. The dissemination of carbapenem resistance in *Enterobacteriaceae* is a major public health concern, as carbapenem antibiotics are a last-resort therapy for these common pathogens.

OXA-427 is a recently described plasmid-mediated transferable CHDL discovered in a *Serratia marcescens* isolate from a university hospital in Leuven, Belgium [Bogaerts et al., 2017]. It was subsequently detected in various *Enterobacteriaceae* species from eight additional clinical isolates. It is a novel carbapenemase that possesses an unusual resistance profile, including broad-spectrum penicillins, extended-spectrum cephalosporins, and carbapenems. In this study, we have structurally characterized this enzyme, providing the possible structural basis for its unusual hydrolytic profile.

RESULTS AND DISCUSSION

Kinetic parameters determination

To evaluate the biochemical properties of OXA-427, steady-state kinetic parameters were determined (Table 1). Overall, the enzyme presents fairly good affinity for representative antibiotics from each family of β -lactams, e.g. benzylpenicillin, cefalotin, ertapenem, and aztreonam. For certain substrates, the determined K_m was unexpectedly high, namely for amoxicillin, oxacillin and imipenem. In the case of oxacillin, biphasic curves were observed, and values could only be determined for the second phase. OXA-427 presents mostly low turnover rates for most substrates, the exception being amoxicillin, ceftazidime, cefepime and imipenem, for which modest turnover rates were observed when compared for example to the 5 s^{-1} turnover rate of OXA-48 with imipenem [Docquier et al., 2009] or 10 s^{-1} OXA-18 for ceftazidime [Dabos et al., 2018]. Catalytic efficiency seems to correlate well with previous studies on OXA-427 (Table 2) [Bogaerts et al., 2017] that determined that, according to EUCAST cut-off values [The European Committee on Antimicrobial Susceptibility.], the enzyme conferred resistance to penicillins, ceftazidime, cefepime, aztreonam, and ertapenem, for which values between 8 and 30 were determined, but showed susceptibility to imipenem, meropenem and cefotaxime, for which values below 2 were observed. Unexpectedly, the catalytic efficiency for ampicillin was rather low. Further studies should determine whether the behaviour of the enzyme changes under different conditions. Kinetic parameters also suggest the enzyme may confer resistance towards cefalotin, but likely does not against oxacillin.

Table 1 Steady-state kinetic parameters of β -lactamase OXA-427.

Substrate	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)
Benzylpenicillin	12	0.247	20.6
Amoxicillin	950	1.216	1.3
Ticarcillin ^a	< 20	0.315	> 15.7
Oxacillin ^b	1,307	0.315	0.2
Piperacillin	17	0.255	15.0
Cefalotin ^a	< 10	0.157	> 15.7
Cefotaxime	25	0.047	1.9
Ceftazidime	65	1.799	27.7
Cefepime	125	3.700	29.6
Ertapenem ^a	< 10	0.083	> 8.3
Meropenem	33	0.037	1.1
Imipenem ^c	> 3,070	> 5.967	1.9
Aztreonam	18	0.356	19.8

^a K_m is below the measurable concentrations

^bBiphasic behaviour, values determined for the second phase

^c K_m is above the measurable concentrations, hydrolysis rate keeps rising, therefore k_{cat} cannot be corroborated, catalytic efficiency is estimated from these values

Table 2 MICs of E.coli DH10B expressing OXA-427.

β -lactam	MICs (mg/L)
Ticarcillin/CLA	>256
Temocillin	512
Piperacillin/TAZ	256
Ceftazidime	>128
Cefotaxime	0.25
Cefepime	32
Aztreonam	128
Imipenem	2
Meropenem	1
Ertapenem	2

CLA: clavulanic acid

TAZ: tazobactam

From [Bogaerts et al., 2017]

OXA-427 crystallization and X-ray crystallography

OXA-427 crystallized with a buffer solution containing 2.2 M ammonium sulfate and 0.2 M sodium fluoride. The structure was determined by molecular replacement using the deposited OXA-45 structure (PDB: 4GN2) as a template, and the model was refined to 2.78 Å (Table 3). The asymmetric unit contains two protein chains, A and B, modelled with 241 residues each. Both chains present the typical class D fold with an α -helical region and a mixed α -helix/ β -sheet region, with a C α RMSD of 0.90 Å compared to the OXA-45 structure (PDB code 4GN2) and 0.84 Å compared to OXA-1 (PDB code 1M6K). 92 % of all residues are inside the favoured regions of the Ramachandran plot, 7% in the allowed regions, and 1% are outliers. Electron density allows for all regions of the protein to be modelled, both backbone and sidechains (Fig. 1). For loop β 4- β 5 on chain A, which is the homolog of the β 5- β 6 loop

Table 3. Crystallography data collection and refinement statistics.

Data collection	
Space group	C 1 2 1
Cell dimensions	
a, b, c (Å)	143.79, 42.58, 99.71
α , β , γ (°)	90.00, 114.37, 90.00
Resolution (Å)	68.10-2.78
R _{meas}	19.9 % (overall) 6.7% (inner shell)
R _{pim}	14.0 % (overall) 4.7% (inner shell)
I/ σ (I)	7.2 (overall) 2.3 (outer shell)
Completeness (%)	94.2
Redundancy	3.00
CC(1/2)	0.982 (overall) 0.708 (outershell)
Refinement	
Resolution range (Å)	68.10-2.78
No. unique reflections	13,454
R _{work} /R _{free}	2035%/26.9%
No. non-hydrogen atoms	
Protein	3,824
Water	134
Ligand/Ions	13
Total	3,971
Average B, all atoms (Å ²)	50.00
Protein	50.46
Water	40.36
Ligand/Ions	79.11
Root mean squared deviations	
Bond lengths (Å)	0.01
Bond angles (°)	1.17

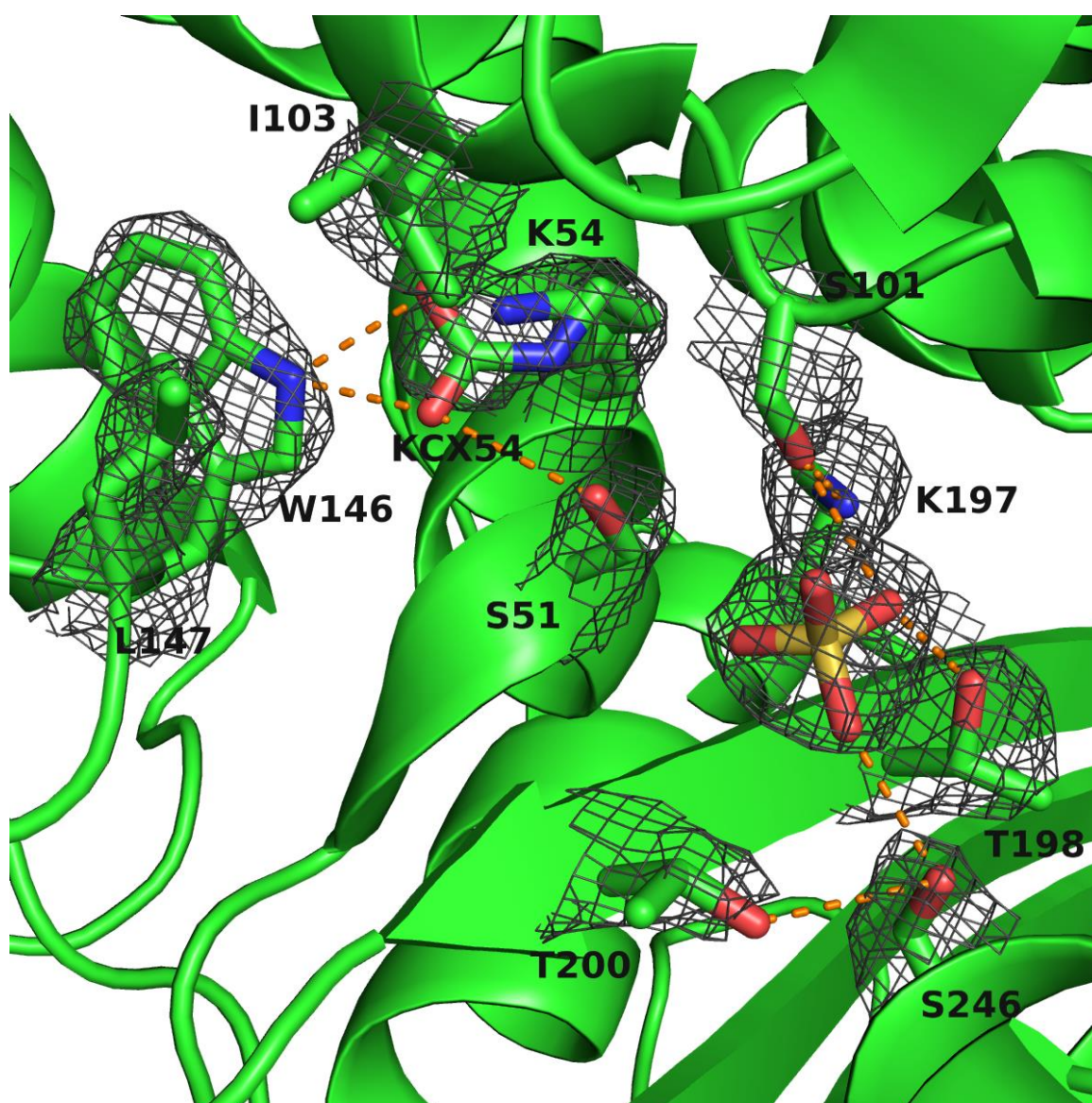


Figure 1. Crystal Structure of OXA-427. Electron density can be seen for all residues in the active site and a sulfate ion is make hydrogen bonds with S101, T198 and S246 in both chains. K54 is partially carbamoylated, showing different conformations in the carbamoylated and non-carbamoylated forms.

of OXA-48, weak electron density allows for the backbone orientation of this loop to be modelled, as well as the general orientation of the sidechains. Several ordered water molecules have been modelled in the structure as well as sulfate ions bound in the active site. No significant differences can be observed between both chains, except for the conformation adopted by the flexible $\beta 4$ - $\beta 5$ and $\beta 6$ - $\alpha 10$ loops. In chain B, the $\beta 4$ - $\beta 5$ loop adopts an elongated conformation, extending away from the protein. In chain A, the same loop folds back towards the Ω -loop. $\beta 6$ - $\alpha 10$ loop adopts a slightly shifted conformation in both chains, closing more on the active site of chain B. This suggests that these two loops, $\beta 4$ - $\beta 5$ and $\beta 6$ - $\alpha 10$, may be quite flexible in OXA-427. The crystal of OXA-427 appears to be only partially carbamoylated, and K54 has been modelled with occupancy of 0.5 for Lys and 0.5 for KCX. KCX54 is stabilized by hydrogen bonds to S51, S106 and W146. Unlike other OXA enzymes, no water molecule is observed in this structure in the proximity of KCX54. Possibly, the presence of asparagine instead of leucine in position 57 is necessary to favour

the positioning of this stabilizing water that may be observed in other OXA structures. As for OXA-1-like enzymes, the Ω -loop of OXA-427 is also longer than in most other class D enzymes. The extra sequence of this loop, farthest from the active site cavity, seems to allow for more interactions with its inner portion, possibly influencing the conformation of the rest of the loop, which seems to come closer to the β_4 strand and the β_4 - β_5 loop than even in OXA-1. This may also occur due to smaller sidechains present in OXA-427, having A50 and S148 in place of D69 and E162 in OXA-1 or M69 and G159 in OXA-45 (Fig. 2). OXA-427

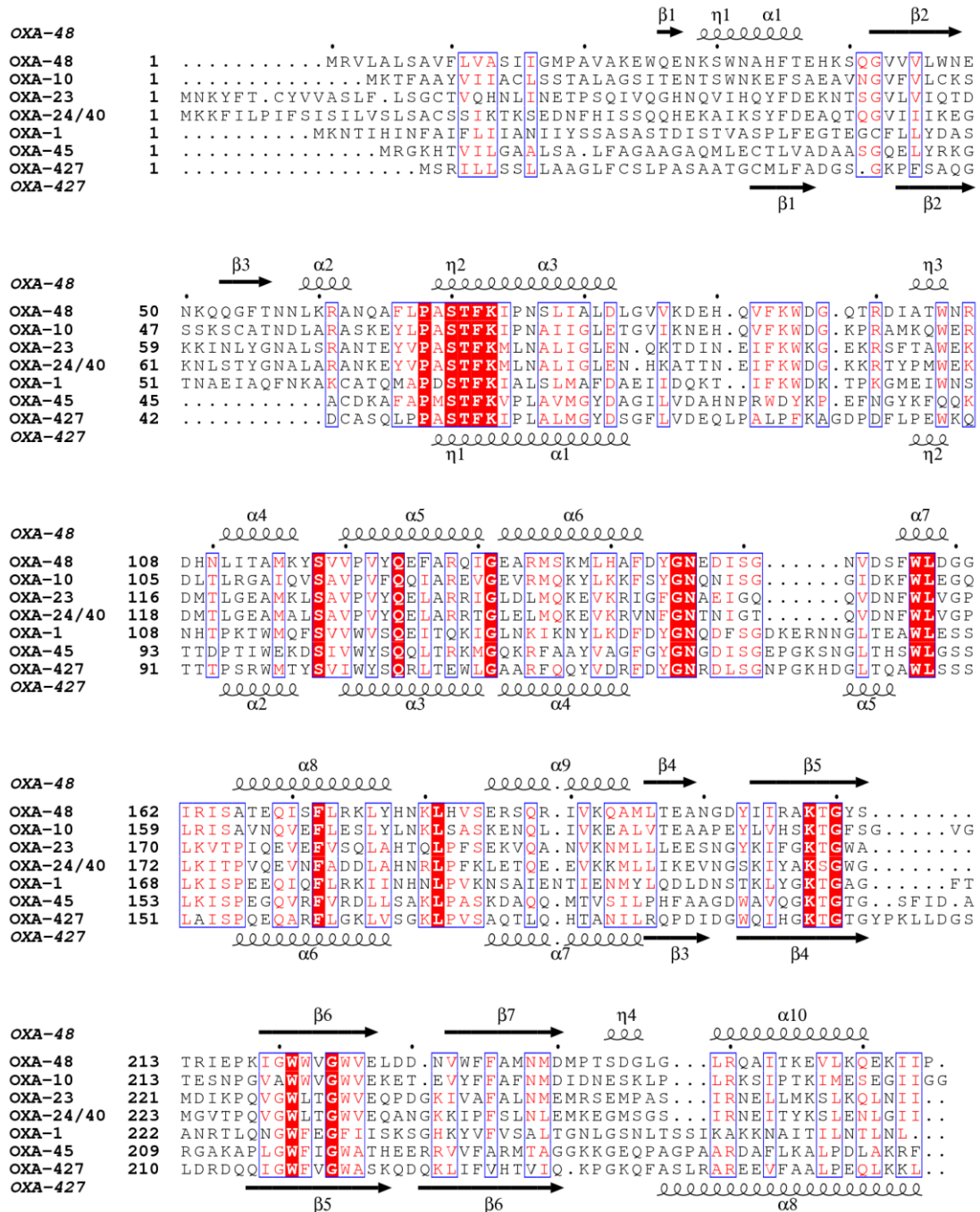


Figure 2. Sequence alignment of OXA-427 with other closely related or representative OXAs. Sequence alignment shows OXA-427 presents the conserved class D motifs. Notice the shorter sequence of OXA-427 and OXA-45 enzymes, that lack the first β -sheet and two α -helices compared to OXA-48.

also presents L147 in an orientation that seems to distance it farther from the β 4- β 5 loop than in other OXAs, and bring it closer to I103 (V114 or V120 in OXA-1 or OXA-48, respectively), and this shift may influence the access of water molecules to the scissile bond [Smith et al., 2013] and the steric impediment of the active site cavity for bulkier substrates such as ceftazidime [Mitchell et al., 2015]. The β 4- β 5 loop itself is much longer than in other OXAs, being 5 and 8 residues longer than in OXA-1-like and OXA-48-like enzymes, respectively. It seems to lean in both chains towards the Ω -loop, with the N-terminal portion with a similar conformation to that seen in the OXA-255/ceftazidime complex (PDB code 4X55). As mentioned before, this may be due to smaller residues present in the interface of both loops, such as A50 right before the SXXK motif, S148 instead of E162 in OXA-1, or G201 at the N-terminus of the β 4 β 5 loop itself, but also the lengthier loop itself probably allows the loop to adopt a more relaxed conformation than in OXAs with shorter loops. OXA-427 belonging to the OXA-12-like family (historically named AmpH or AmpS. It is closest to OXA-1 that to other important OXA families, and possesses no R250 beneath the active site cavity to make a salt bridge with the C3/C4 carboxylate of β -lactams. Instead, S246 at the N-terminus of the α 10 helix, together with T198 from the KTG motif would probably make hydrogen bonds with the carboxylate upon interaction with the substrate, as in OXA-1-like enzymes. Being a class D enzyme, OXA-427 has a mostly non-polar active site cavity, and apparently presents a kind of hydrophobic bridge closing the active site cavity (Fig. 3). The bridge on OXA-427 would be formed, on one side, by L85, in the same position

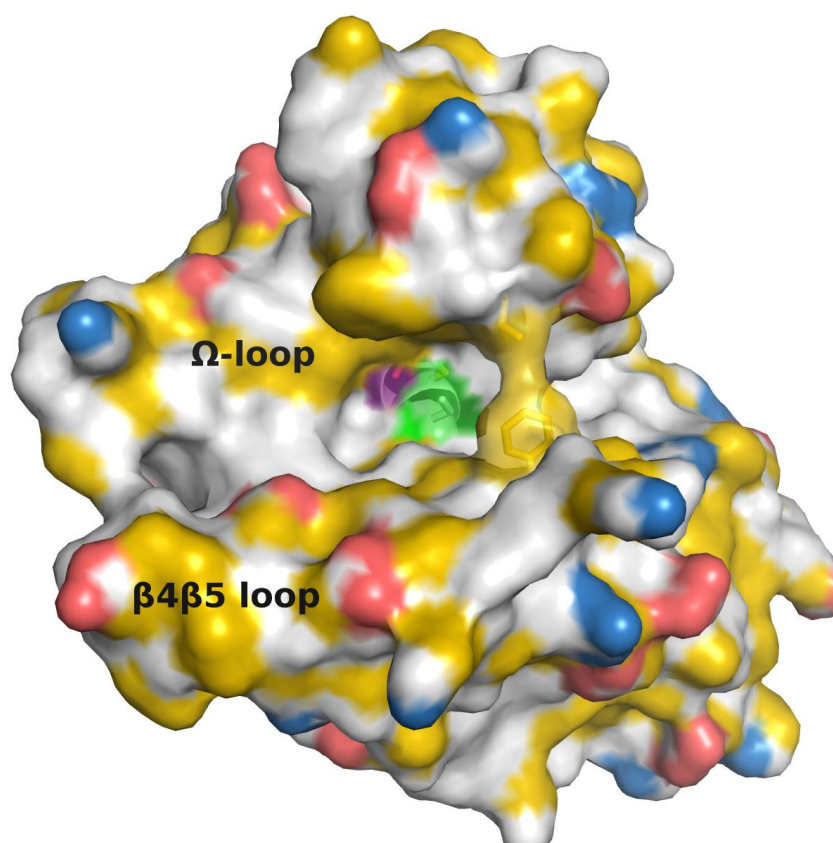


Figure 3. Hydrophobic bridge in the OXA-427 active site cavity. The active site cavity of OXA-427 can be seen colored in red for positively charged surfaces, blue for negatively charged surfaces, and yellow for hydrophobic surfaces. A hydrophobic bridge is formed between L185 and F244, whose surfaces are shown transparent. For reference, the Ω -loop and β 4 β 5-loop are labelled, and S51 and KCX54 are shown in green and purple, respectively.

as Y/F112 in OXA-23 or OXA-24/40 enzymes. On the other side, unlike OXA-23-like or OXA-24/40-like enzymes, it is not a residue from the β 4- β 5 loop analogue to M221 that closes the bridge in OXA-427, but F244, at the N-terminus of the α 10 helix, right before S246. This would make the bridge in OXA-427 close the active site cavity groove farther out than those of OXA-23-like or OXA-24/40-like enzymes.

Molecular dynamics simulations and water network analysis

A 10 ns molecular dynamics (MD) simulation of apo OXA-427 shows that certain parts of the enzyme present large RMS fluctuations (Fig. 4). Regions showing the highest flexibility comprise the distal extra part of the expanded Ω -loop, the β 4- β 5 loop, the β 6- α 10 loop comprising F244, and the C-terminus of the enzyme. Other regions show a lesser degree of flexibility, such as the loop comprising the L85 residue that closes the hydrophobic bridge. The flexibility of F244 in the β 6- α 10 loop would allow the active site to bind the substrates, and the lesser flexibility of the L85 loop suggests that substrates that would overlap with L85 probably present a higher binding energy cost than those that may displace F244.

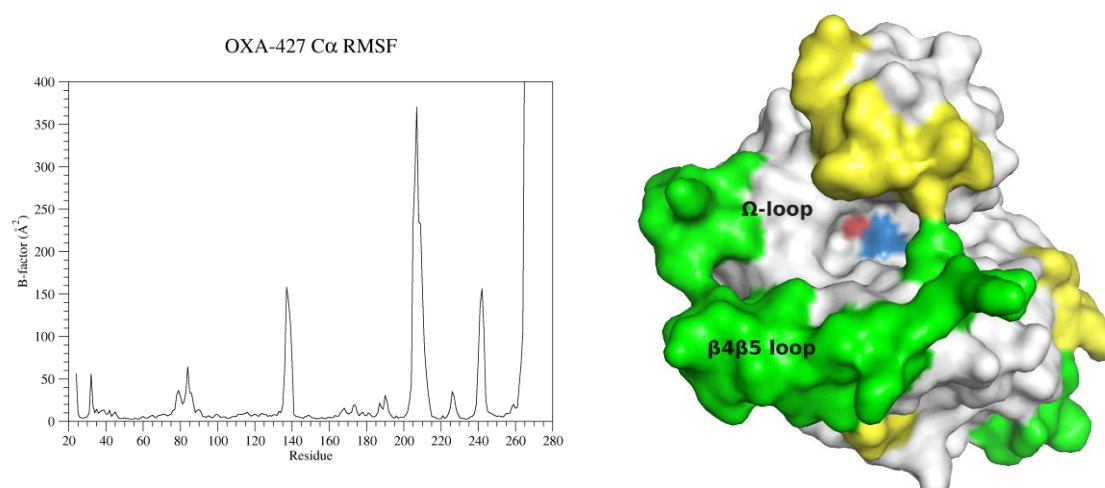


Figure 4. OXA-427 flexibility. A – left) Protein C α RMSF plot showing the protein residues with larger flexibility. Most flexible regions comprise the loops around the active site cavity. B – right) 3D representation of OXA-427 with the most flexible regions colored in green, namely the β 4- β 5 loop, the Ω loop and the β 6- α 10 loop comprising the F244 among other residues. Regions showing a lesser degree of flexibility are colored in yellow, such as the loop comprising L85. S51 and KCX54 are colored blue and red, respectively.

HOP water network analysis shows a more elaborate network of waters inside that active site cavity than those observed in the crystal structure (Fig. 5). Two hydration sites are found overlapping the same region occupied by the sulfate anion in the crystal structure. The water molecules binding close to the oxyanion hole and S148 are also found by HOP analysis. Certain waters like the one binding T99 and the sulfate anion are not found by HOP analysis, which suggests that it was stabilized by the sulfate anion, which was not included in the MD simulation. The water network found by HOP analysis also extends further inside the active site cavity starting from the oxyanion hole and S148, S51, KCX and W146. A water molecule is observed in the MD simulation behind the KCX carbamoyl, which was not found in the crystal structure.

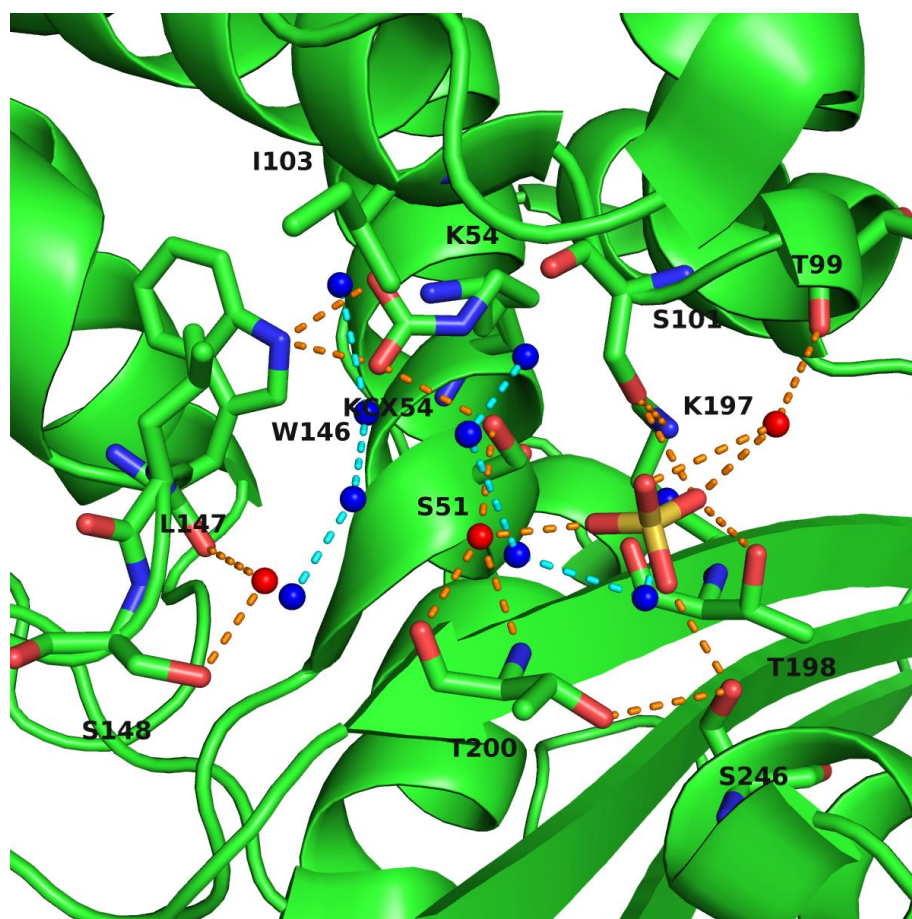


Figure 5 – OXA-427 active site cavity water network. Comparison between the water network observed in the active site cavity of OXA-427 in the crystal structure and the one evidenced by water HOP analysis of the MD simulation. Certain spaces filled by waters in the crystal structure (red spheres) show similarly positioned waters in the HOP analysis (blue spheres). Notice the two water molecule positions predicted by HOP analysis close to two of the oxygen atoms of SO_4^{2-} in the crystal structure.

Structural comparison with β -lactams complexed with other OXA enzymes

The OXA-427 structure was superposed on different class D β -lactamase covalent complexes with substrates to analyse how they might interact in its active site. Superposition of the OXA-1/oxacillin complex on OXA-427 shows that the R1 substituent of oxacillin would have severe overlaps with residues forming the hydrophobic bridge on OXA-427, and especially with L85 (Fig. 6). Clashes with the S101 sidechain are also observed, but this sidechain can turn, as observed in other class D structures, to participate in the catalytic mechanism.

Superposition of the OXA-225/ceftazidime complex (PDB code 4X55) shows that light overlaps would occur between the R1 substituent of ceftazidime and the sidechains of T200 and F244, but also the R2 sidechain, which is still present in ceftazidime in the 4X55 structure, would present serious clashes with F244 (Fig. 7).

The differences in K_m for these two substrates however, may be due to the fact that the clashing sidechain in the case of oxacillin is not lost upon acylation and would clash against the relatively stable L85, whereas in the case of ceftazidime, the most serious clashes are due to the R2 leaving group, that is normally lost during acylation, and occur against F244, which

appears to be more flexible than L85 and probably contributes more to the opening of the hydrophobic bridge for ligand binding.

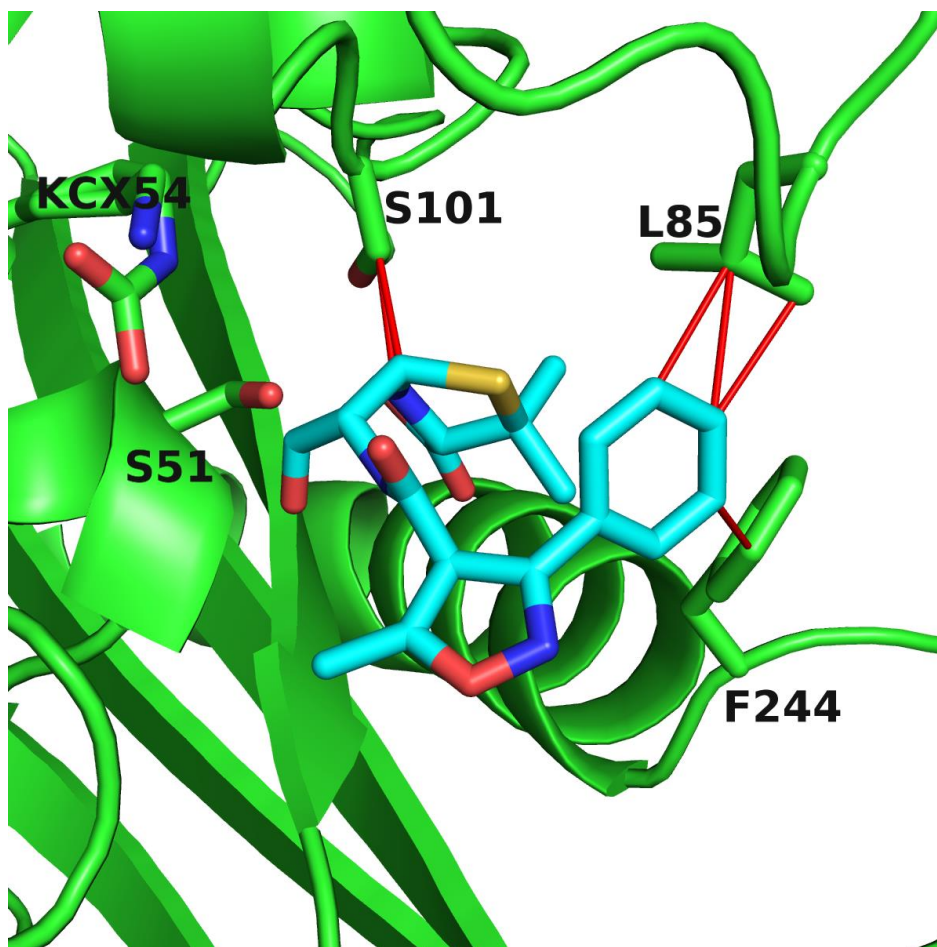


Figure 6. Superposition of OXA-1/oxacillin complex on the OXA-427 structure. The R1 phenyl ring has severe overlaps with the hydrophobic bridge-forming residues in OXA-427, especially with L85.

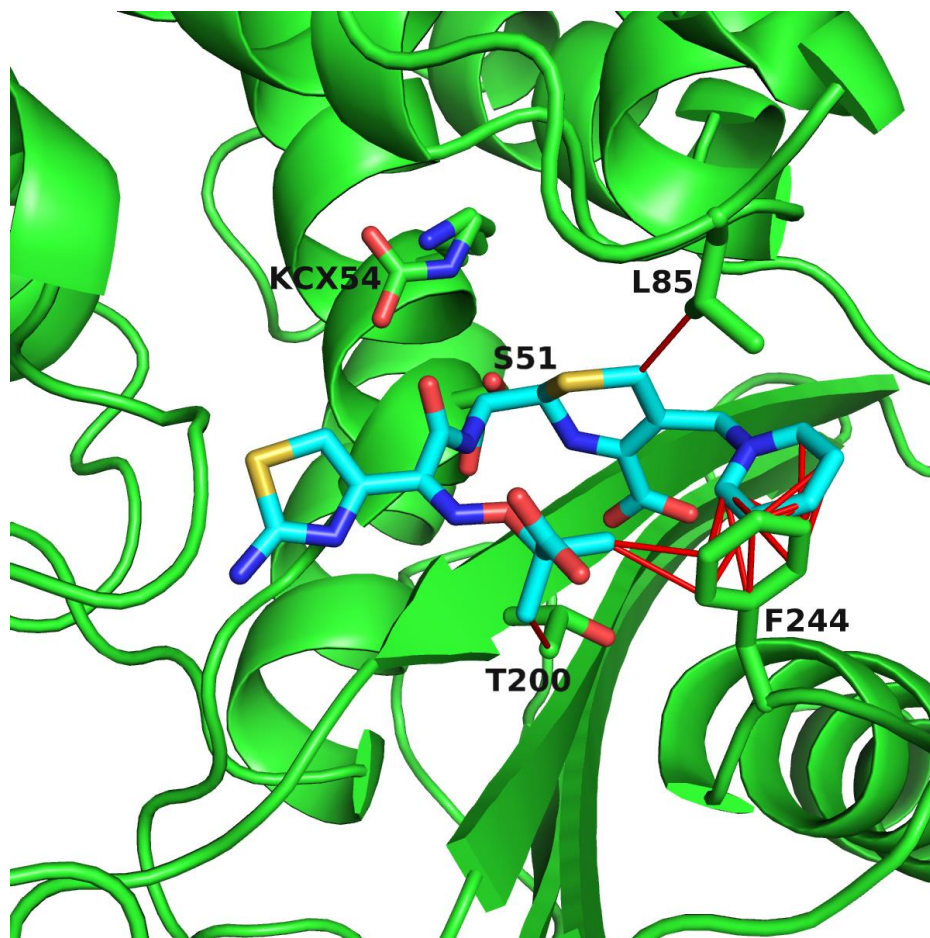


Figure 7. Superposition of OXA-255/ceftazidime complex on the OXA-427 structure. Serious overlaps would occur between the R2 substituent leaving group that is still present in the OXA-255/ceftazidime crystal structure, and F244 from the hydrophobic bridge. Minor clashes would be present between the R1 substituent with F244 and T200, and the dihydrothiazine ring with L85.

CONCLUSION

We determined the crystal structure of OXA-427, which shows a classical class D fold, but with peculiar structural features not observed in many class D families. An extended Ω -loop and $\beta 5$ - $\beta 6$ loop is characteristic of OXA-1-like and related families, but the analogue $\beta 4$ - $\beta 5$ loop of OXA-427 is much longer. The active site KCX seems to be only partially carbamoylated, without the presence of an inhibitor or acidic pH in the crystallization conditions, suggesting a less stable post-translational modification in this enzyme than in other class D enzymes. A hydrophobic bridge seems to close the active site cavity, but at a different position than in OXA-23-like or OXA-24/40-like enzymes, enclosing a deeper active site cleft. This may provide a good explanation for the relatively wide hydrolytic profile showed by this enzyme, conferring resistance or diminished susceptibility to members of each of the β -lactam families [Bogaerts et al., 2017], including aztreonam, bulky cephalosporins such as ceftazidime, or large carbapenems like ertapenem. Further kinetic parameters determination, co-crystallization studies and molecular modelling experiments are needed to complement these preliminary data and better explain the peculiar hydrolytic spectrum of this enzyme.

EXPERIMENTAL PROCEDURES

Bacterial strains

*bla*_{OXA-427} carrying strains were obtained from previous studies [Bogaerts et al., 2017]. *E. coli* TOP10 (Invitrogen, Saint-Aubin, France) and *E. coli* BL21 (DE3) (Novagen, VWR International, Fontenay-sous-Bois, France) were used for cloning and protein overproduction, respectively.

Cloning of *bla*_{OXA-427} gene

PCR amplification of *bla*_{OXA-427} was performed using total DNA extraction of *E. coli* DH10B clone 2.9 [Bogaerts et al., 2017], and primers INFampSFw (5'-aaggagatatacatatgtcccgattctgttatccagcct-3') and INFampSRv (5'-gggtgggtgctcgaccagtttcttcagctgttcgggcagtg-3'). Amplicons were cloned into pET-41b(+) expression vector (Novagen, VWR International, Fontenay-sous-Bois, France) using the NEBuilder® HiFiDNA Assembly Cloning Kit (New England BioLabs® Inc, United Kingdom), following the manufacturer's instructions. Recombinant plasmid was electroporated into *E. coli* TOP10 and selected using TSA-plates containing kanamycin (50 mg/L). Recombinant plasmid was extracted using the Qiagen miniprep kit and both strands of the insert were sequenced using T7 promoter and T7 terminator primers, for pET-41b(+) (Novagen, VWR International, Fontenay-sous-Bois, France), with an automated sequencer (ABI Prism 3100; Applied Biosystems, Les Ulis, France). The nucleotide sequences were analysed using software available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). Recombinant plasmid pET41b-OXA-427 was electroporated into electrocompetent *E. coli* BL21 (DE3) and selected using TSA-plates containing kanamycin (50 mg/L).

β-Lactamase purification

Overnight cultures of *E. coli* BL21 (DE3) harbouring pET41b-OXA-427 were used to inoculate 2 L of BHI broth containing 50 mg/L kanamycin. Bacteria were cultured at 37°C until reaching an OD of 0.6 at 600 nm, and protein expression was induced overnight at 25°C with 0.2 mM IPTG. The culture was then centrifuged at 6000 g for 15 min and the pellets resuspended with 10 mL of Buffer A (20 mM PBS, 175 mM K₂SO₄, 40 mM imidazol, pH 7.40). Bacterial cells were disrupted by sonication and protein solution was clarified by centrifugation at 10,000 g for 1 h at 4°C. The supernatant was then centrifuged at 48,000 g for 1 h at 4°C. OXA-427 was purified using a NTA-Nickel pseudo-affinity chromatography column (GE Healthcare, Freiburg, Germany). Elution was performed in a gradient of 0 to 100% Buffer B (20 mM PBS, 175 mM K₂SO₄, 500 mM imidazol, pH 7.40). Purity was assessed by SDS-PAGE, and pure fractions were pooled and dialyzed against 100 mM sodium phosphate buffer (pH 7.4) 50 mM potassium sulphate and concentrated up to 6.9 mg/ml using Vivaspin® columns (GE Healthcare, Freiburg, Germany). Protein concentration was determined using Bradford Protein assay (Bio-Rad) [Bradford, 1976].

Steady-state kinetic parameters

Kinetic parameters of purified OXA-427 were determined at 100 mM sodium phosphate buffer (pH 7.0). The k_{cat} and K_m values were determined by analysing hydrolysis of β-lactams under initial-rate conditions with an ULTROSPEC 2000 model UV spectrophotometer (Amersham Pharmacia Biotech) using the Eadie-Hoffstee linearization of the Michaelis-Menten equation. The different β-lactams were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Protein crystallization and X-ray crystallography

Conditions for OXA-427 crystallization (6.9 mg ml⁻¹) were identified from screening stochastic crystallization conditions among the commercially available suites: Classics, AmSO₄, PEGs and PEGs II (Qiagen/NeXtal), using the Mosquito® HTS (TTP LabTech). The tiny crystal was transferred to a solution consisting of the mother liquor supplemented with 25% glycerol as cryoprotectant and flash-frozen in liquid nitrogen. Diffraction data were collected at 100 K at a wavelength of 0.980 Å on the PROXIMA2 beamline at the SOLEIL synchrotron (Saint-Aubin, France). They were re-indexed, integrated and scaled using the autoPROC toolbox [Vonrhein et al., 2011], implementing XDS for the two first steps and AIMLESS [Evans and Murshudov, 2013] for the third one. The structure of OXA-427 with two molecules per asymmetric unit was successfully solved using the automatic molecular replacement program *MrBUMP* [Keegan et al., 2007] with OXA-45 (PDB entry 4GN2; 41% sequence identity) as a search model. The model was rebuilt manually in Coot [Emsley et al., 2010] and then refined using BUSTER-TNT [Bricogne et al., 2011] with local noncrystallographic symmetry (NCS)

restraints and a translation–libration–screw (TLS) description of B factors [Murshudov et al., 2011]. The quality of the final refined model was assessed using MolProbity [Chen et al., 2010]. Data collection and refinement statistics are given in Table 3. Crystal structure images were generated using PyMOL (<http://www.pymol.org>) [Schrödinger, 2015] and the YRB script [Hagemans et al., 2015].

Structure analysis and molecular dynamics simulations

OXA-427 structural analysis and comparison with other crystal structures were performed with UCSF Chimera package [Pettersen et al., 2004]. Molecular dynamics simulations of OXA-427 were performed with Gromacs version 4.6 [Pronk et al., 2013] using the OPLS-AA force field [Kaminski et al., 2001]. HOP software version 0.4.0 alpha 2 (<https://github.com/Becksteinlab/hop>) [Beckstein et al., 2009] was used for water molecule dynamics analysis. PyMol was used to generate images [Schrödinger, 2015].

PDB deposition

The crystallographic structure of OXA-427 has been deposited to the PDB, accession code 6HUH. Authors will release the atomic coordinates and experimental data upon article publication.

ACKNOWLEDGEMENTS

We acknowledge SOLEIL for provision of synchrotron radiation facilities (proposal ID BAG20150780) in using PROXIMA beamlines. This work was supported by the Laboratory of Excellence in Research on Medication and Innovative Therapeutics (LERMIT) [grant number ANR-10-LABX-33], by the JPIAMR transnational project DesInMBL [grant number ANR-14-JAMR-0002] and by the Région Ile-de-France (DIM Malinf).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interests with the content of this article.

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Structural plasticity of class D beta-lactamases: OXA-517, a novel OXA-48 variant with carbapenem and expanded spectrum cephalosporin hydrolysis.

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Abstract

OXA-48-producing *Enterobacteriaceae* have now widely disseminated throughout European countries. Since the first identification of OXA-48, a number of variants have been reported, differing by just a few amino acid substitutions or deletions, mostly in the region of loop β 5- β 6. Here we have characterized the first OXA-48-like β -lactamase, named OXA-517, capable of hydrolyzing at the same time extended-spectrum cephalosporins and carbapenems, identified in a multidrug-resistant *Klebsiella pneumoniae* strain 1219, recovered in Belgium. OXA-517 presents a substitution in Arg214Lys and a deletion of Ile215 and Glu216 in the β 5- β 6 loop. According to MICs values, this protein harbors hydrolytic activity against expanded-spectrum cephalosporins as well as carbapenems. The substrate specificity was confirmed by steady state parameters of the purified enzyme, which exhibited high catalytic efficiencies for expanded-spectrum cephalosporins and carbapenems. The *bla*_{OXA-517} gene coding for OXA-517 was located on a ca. 31-kb plasmid identical to the prototypical IncL *bla*_{OXA-48}-carrying plasmid except for a ca. 30.7-kb deletion in the *tra* operon, and for the insertion of *IS1R*. The crystal structure of the OXA-517 carbapenemase, determined to 2.1 Å resolution for a crystal grown at pH 6.5, reveals an expanded active site as compared to that of OXA-48, which allows the bulky substrate ceftazidime to be accommodated. At the same time, according to docking analysis, the meropenem carbonyl is bound in the oxyanion hole, forming hydrogen bonds with the amide NH groups of Tyr211 and Ser70, facilitating the hydrolysis of carbapenems.

- For be submitted to EMBO Molecular Medicine.

Introduction

Antimicrobial resistance is the most alarming emerging problem in infectious diseases. β -Lactams, due to their safety, reliable killing properties and clinical efficacy, are among the most frequently prescribed antibiotics used to treat bacterial infections. However, their utility is being threatened by the worldwide proliferation of β -lactamases (BL) with broad hydrolytic capabilities (Poirel et al, 2012a). Currently, BL-mediated resistance does not spare even the most powerful β -lactams (i.e. carbapenems), whose activity is challenged by carbapenemases (Queenan & Bush, 2007). OXA-48, a class D carbapenemase (CDHLs) initially identified in a carbapenem-resistant *Klebsiella pneumoniae* isolate from Turkey in 2001, has since spread globally in *Enterobacteriaceae* (Poirel et al, 2010; Poirel et al, 20015). Several variants have since been reported, differing by a few amino acid substitutions or deletions (Poirel et al, 2010; Poirel et al, 20015; Oueslati et al, 2015). For a complete list of variants see the Beta-Lactamase DataBase (Naas et al, 2017) (<http://bldb.eu/BLDB.php?class=D#OXA>). OXA-48 hydrolyzes penicillins at high level, carbapenems at low level and lacks significant expanded-spectrum cephalosporin (3GC) hydrolase activity (Poirel et al, 2012b). Whereas some OXA-48-variants with single amino acid substitutions have similar hydrolytic activities as OXA-48, others, such as OXA-163 or OXA-405, have a four-amino-acid deletion that results in the loss of carbapenem hydrolysis and gain of expanded-spectrum cephalosporin hydrolysis (Poirel et al, 2011; Dortet et al, 2015).

The mechanism of hydrolysis of β -lactam antibiotics by OXA-48 and other class

Table 1. MICs of β -lactam antibiotics for *K. pneumoniae* 1219, *E. coli* TOP 10 (pOXA-517), *E. coli* TOP 10 (pTOPO-OXA-48), *E. coli* TOP 10 (pTOPO-OXA-517),

Antibiotic	MIC (mg/L)					
	<i>K. pneumoniae</i> 1219	<i>E. coli</i> TOP10				<i>E. coli</i> TOP10
		pOXA-517	pTOPO-OXA-48	pTOPO-OXA-517	pTOPO-OXA-163	
Amoxicillin	>256	>256	>256	>256	>256	2
Amoxicillin + CLA ^a	192	64	192	64	96	2
Piperacillin	>256	>256	128	>256	>256	1.5
Piperacillin + TZB ^b	96	96	12	96	32	1
Cefotaxime	>32	2	0.094	0.38	3	0.06
Ceftazidime	48	16	0.19	4	16	0.12
Ceftazidime+Avibactam ^c	1	1	0.5	0.5	ND	0.25
Cefepime	12	1	0.047	0.25	0.5	0.023
Imipenem	8	0.75	0.38	0.38	0.25	0.25
Meropenem	3	0.125	0.047	0.047	0.023	0.016
Ertapenem	3	0.125	0.047	0.047	0.032	0.003
Doripenem	3	0.094	0.094	0.094	0.023	0.06
Temocillin	192	192	>1024	32	32	4
Aztreonam	4	2	0.094	0.75	2	0.047

E. coli TOP 10 (pTOPO-OXA-405), *E. coli* TOP 10 (pTOPO-OXA-163), and *E. coli* TOP 10.

D β -lactamases (DBLs) proceeds via acylation and deacylation of the active-site serine and involves a carboxylated lysine as the general base (Golemi et al, 2001; Li et al, 2015). This reversible lysine modification, which is essential for DBL-activity, is proposed to be a spontaneous reaction, facilitated by the hydrophobic environment of the active site and dependent on the protonation state of the lysine and availability of CO₂ (Leonard et al, 2013; Golemi et al, 2001; Baurin et al, 2013).

Despite a remarkable sequence divergence with other DBLs, the tertiary structure of OXA-48 is very similar to that of other DBLs (Docquier et al, 2009; Iorga, personal communication). One notorious difference is that the β 5 and β 6 strands, located close to the substrate binding site, are overall shorter in OXA-48 than in other DBLs. (Docquier et al, 2009). As a consequence, the β 5– β 6 loop has a different length and orientation from that found in other DBLs. The observed conformation of the loop in OXA-48 is promoted by a salt bridge of Arg214 with the Ω -loop Asp159 residue (Docquier et al, 2009). The β 5– β 6 loop extends into the outer portion of the active site crevice and defines a rather

hydrophilic cavity filled with several water molecules, which are the best candidates to perform the nucleophilic attack on the acyl-enzyme intermediate in the deacylation step (Docquier et al, 2009).

In OXA-163, an OXA-48-like enzyme that hydrolyzes expanded-spectrum cephalosporins instead of carbapenems, the $\beta 5$ – $\beta 6$ loop is shorter because of the R₂₁₄LEP₂₁₇ deletion that results in an expanded active-site cavity compared to that of OXA-48 (Stojanoski et al, 2015). Arg214 is within the four-amino acid deletion, which eliminates one boundary of the active site and elongates the groove. As Arg214 has been suggested to form electrostatic interactions with carbapenem substrates to facilitate their hydrolysis, this could explain the low activity of OXA-163 toward carbapenems (Docquier et al, 2009). Additionally, this expansion of the active site of OXA-163 is consistent with the ability of the enzyme to accommodate a larger substrate such as ceftazidime (Stojanoski et al, 2015). The hypothesis that Arg214 contributes to the inability of OXA-48 to accommodate ceftazidime is additionally supported by the observation that a shorter and uncharged side chain at position 214 (Arg214Ser substitution) results in an increased ceftazidime hydrolysis activity by OXA-232, an OXA-48-like enzyme (Dortet et al, 2015).

The aim of the present study was to characterize OXA-517, a novel OXA-48-variant capable of hydrolyzing carbapenems and expanded-spectrum cephalosporins, identified in a clonal *K. pneumoniae* isolate recovered in Belgium.

Results

K. pneumoniae 1219 clinical isolate (KP 1219) was recovered from culture of a sputum specimen of an 81-year-old woman hospitalized at the AZ Glorieux hospital (Ronse, Belgium) on September 14th, 2015. This patient had been admitted to the emergency ward of this hospital on September 3rd 2015 with rapid alteration of general status,

fever (39°C) and lumbar pain shown to be associated with a hydro-ureteronephrosis due to an obstructive renal stone for which stenting of the left ureteral orifice was performed in the operative room on the next day. Blood and urine cultures obtained on admission revealed the presence of a *Proteus mirabilis* (wild-type susceptible to all antibiotics). Antimicrobial therapy was initiated with temocillin (2 g/IV bid) and rapidly switched to piperacillin 4 g/IV qid. Because of rapid alteration of the status and development of a severe septic shock (thrombocytopenia, 3.000 platelets/mm³) with acute renal failure, (estimated GFR (glomerular filtration rate) of 21 ml/min). The patient had to be admitted to the intensive care ward where she received high dose of vasopressor drugs (norepinephrine 1 µg/kg/min) and was intubated and mechanically ventilated. Her stay was complicated by the development of a right lower lobe ventilator-associated pneumonia episode for which she was treated with several antimicrobial agents including meropenem (1g/IV tid) followed by cefepime (2g/IV tid), colistin (4.5 million units/bid after a loading dose of 9 million units) and tigecycline (100 mg/IV bid). She then developed an acute infectious enterocolitis with active gross bleeding due to *Clostridium difficile* for which she was treated by metronidazole (500 mg tid) and vancomycin (250 mg qid) per os. The patient was nursed in single isolation room during the remaining duration of her ICU stay. After four weeks, she was transferred to another unit for palliative care and eventually died shortly after. *K. pneumoniae* 1219 isolate was repeatedly isolated over the period from throat, sputum, tracheobronchial aspirates and BAL (bronchoalveolar lavage) specimens of the patient.

Using broth microdilution (Sensititre), *K. pneumoniae* 1219 was resistant to amoxicillin and piperacillin (MICs >256 mg/L) and association with clavulanic acid or tazobactam did not restore penicillin-activity (amoxicillin/clavulanic acid >128 mg/L and piperacillin/tazobactam >128 mg/L). The strain was also resistant to expanded-spectrum

Biochemical tests based on imipenem hydrolysis (Carba NP and BYG Carba tests) gave positive results. Although the resistance phenotype was compatible with the production of an OXA-48-like enzyme, the OXA-48 K-SeT[®] assay yielded a negative result. On the other hand, OXA-163-like K-Set assay, another immunochromatographic test specifically targeting the OXA-163-like enzyme, yielded a positive result.

In order to see whether other commercially available tests may be able to detect this possible carbapenemase, Maldi-TOF (Star BL, Bruker) and β -carba (BioRad) were used giving positive results. Similarly, with an inoculum of 10^3 CFU, *K. pneumoniae* 1219 strain grew in chromID® ESBL (BioMérieux) and on the CARBA side of chromID® CARBA SMART (BioMérieux), but not on the OXA-48 side. Finally, phenotypic based tests (eg. CIM or rCIM) clearly identified this isolate as CPE (Gauthier et al, 2017; Muntean et al, 2018).

PCR and sequencing revealed the presence of a new *bla*_{OXA-48} gene variant, designated *bla*_{OXA-517} gene. *bla*_{OXA-517} gene differed from the *bla*_{OXA-48} gene by a 6bp deletion, which included 2 nucleotides of the codon coding for Arg214, the entire codon coding for Ile215 and one nucleotide

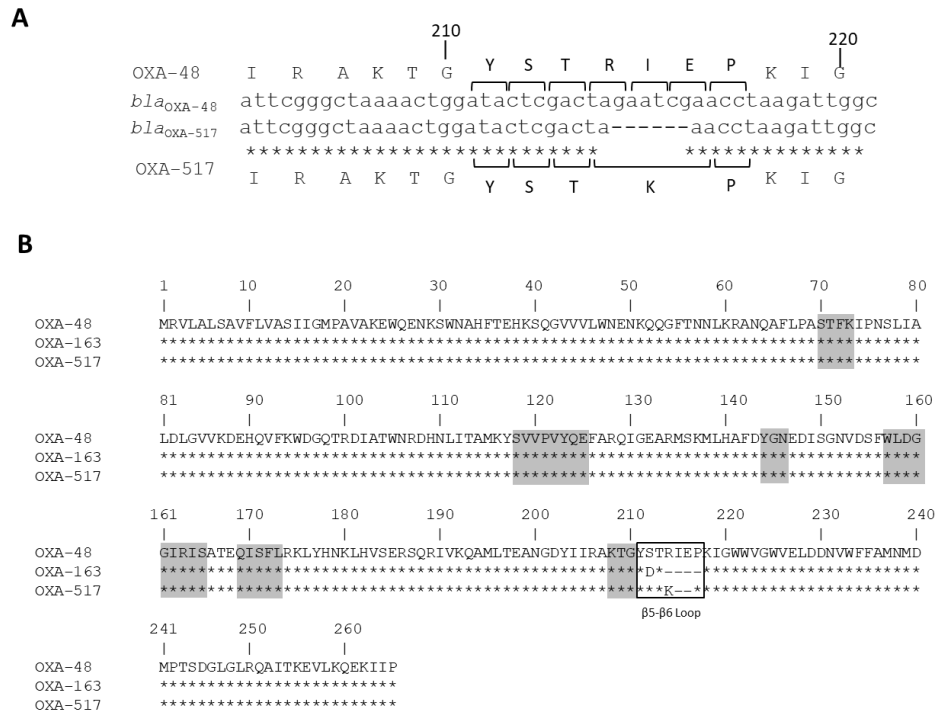


Figure 1. (A) Nucleotide alignment of the β 5- β 6 loop region of the *bla*_{OXA-48} and *bla*_{OXA-517} genes (small letters). The corresponding amino-acid sequences of OXA-48 and OXA-517 are shown in capital letters. **(B)** Alignment of the amino acid sequences of OXA-48, OXA-163 and OXA-517. Asterisks indicate identical residues in all three sequences. Dashes indicate deleted amino acid residues. Amino acid motifs that are well conserved among class D β -lactamases are highlighted in gray. The β 5- β 6 loop sequence is boxed.

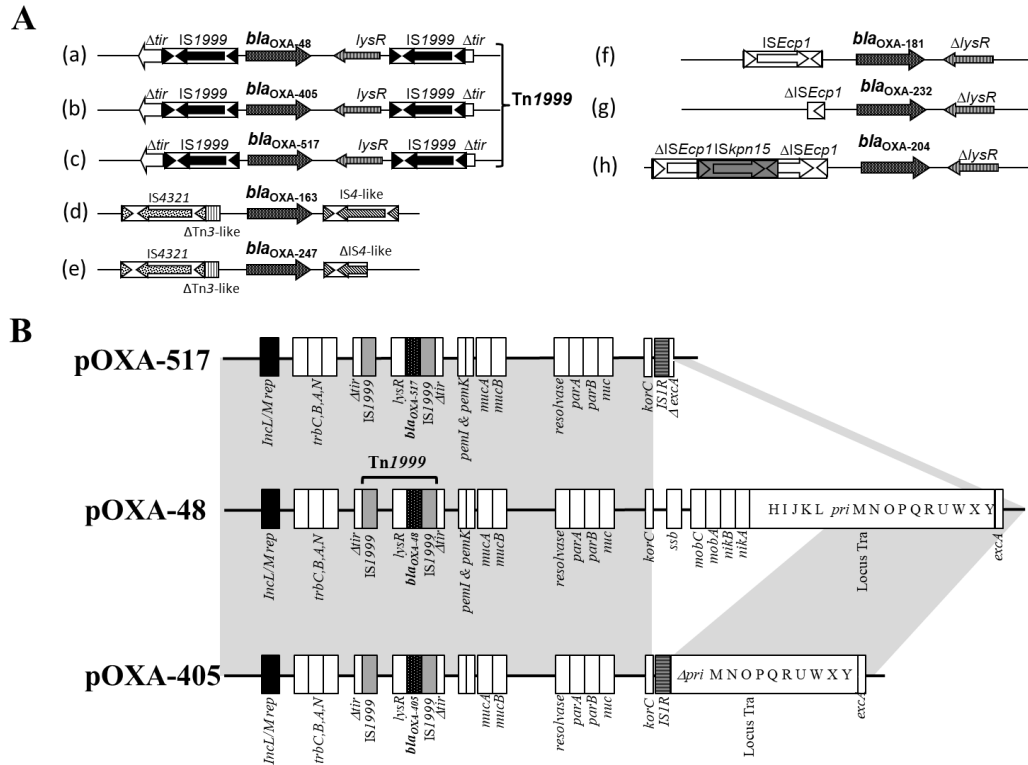


Figure 2. (A) Plasmids structure and genetic environment. Schematic representation of the genetic environment of the *bla*_{OXA-48} (a), *bla*_{OXA-405} (b), *bla*_{OXA-517} (c), *bla*_{OXA-163} (d), *bla*_{OXA-247} (e), *bla*_{OXA-181} (f), *bla*_{OXA-232} (g), and *bla*_{OXA-204} (h) genes. The Tn1999 composite is formed by two copies of the IS1999 insertion sequence, bracketing a fragment containing the *bla*_{OXA-48}, *bla*_{OXA-405} and *bla*_{OXA-517} genes. **(B)** Comparison of the major structural features of the plasmid pOXA-517 from *K. pneumoniae* 1219 with the prototype IncL *bla*_{OXA-48} plasmid pOXA-48 (GenBank accession number JN626286) and pOXA-405. Common structures are highlighted in gray.

of the codon coding for Glu216. With this deletion, a novel codon was created coding for a Lys in position 214, while Ile215 and Glu216 were deleted (Figure 1).

Genomic features of *K. pneumoniae* 1219. *K. pneumoniae* 1219 presented a genome of 5,339,000 bp with a mean coverage of 90.7 X. The Multi locus sequence typing (MLST) of *K. pneumoniae* 1219 according to MLST 1.8 software (Larsen et al, 2012) (<https://cge.cbs.dtu.dk/services/>) revealed ST-188 (3-1-1-3-4-28-39), an ST type rarely described in hospital settings associated with carbapenemase

or ESBL genes (Rodrigues et al, 2016; Zhou et al, 2016; Ewers et al, 2014).

To identify the resistance phenotype of this isolate, resistome was analyzed by searching acquired genes and point mutations involved in resistance (Zankari et al, 2012). The naturally-occurring *bla*_{SHV-37} gene, the acquired *bla*_{TEM-1} gene and the *bla*_{OXA-517} carbapenemase gene, were identified in this isolate. *K. pneumoniae* 1219 also contained an *aph*(3')-Ic gene conferring resistance to kanamycin and neomycin (Ramirez & Tolmasky, 2010), *strA* and *strB* conferring resistance to streptomycin (Sundin, 2002), *sul1*, *sul2* genes conferring resistance to sulphonamides (Skold,2000) and the dihydrofolate reductase gene

(*dfrA14*) conferring resistance to trimethoprim (Bossé et al, 2015). In addition, *oqxA* and *oqxB* genes were also present in the isolate, conferring reduced susceptibility to fluoroquinolones (MIC of 0.5 µg/ml to cipro by microdilution, which is still in the S range for CLSI and EUCAST) (Rodríguez-Martínez et al, 2013). *fos A* gene conferring resistance to fosfomycin was present too in the genome (Fillgrove et al. 2003).

According to plasmid finder (Carattoli et al, 2014), different plasmid replication origins belonging to the incompatibility groups, IncFIB, IncX3, ColRNAI, IncL/M and IncFII were identified. The *bla*_{OXA-517} gene was carried by the IncL-type plasmid as described previously for the *bla*_{OXA-48-like} carbapenemase genes, except for *bla*_{OXA-181} and *bla*_{OXA-232} genes (Potron et al, 2011; Potron et al, 2013; Poirel et al, 2012; Carattoli et al 2015).

Genetic support and environment of *bla*_{OXA-517} gene. The *bla*_{OXA-517} gene was located on a Tn1999 composite transposon (Fig. 2A), as described for *bla*_{OXA-48} gene (Poirel et al, 2012b). Reconstruction of the plasmid using WGS data revealed that it belonged to the IncL incompatibility group. The pOXA-517 plasmid was identical to the prototypical pOXA-48 plasmid of 61,881-bp (Poirel et al, 2012b) accession number JN626286) except for a 31,488-bp deletion from nucleotide 31,191 to 21 according to the pOXA-48 plasmid numbering. This deletion includes *ssb* gene, *mobC* and *mobA* genes; *nikB* and *nikA* genes, all the Locus *Tra*, and part of *excA* genes that were replaced by an insertion sequence *IS1R* (Fig 2B). Direct transfer of the β-lactam resistance marker into *E. coli* J53 by mating-out experiments failed with pOXA-517, confirming that this deletion impacted the transfer ability of pOXA-517. Electroporation of this plasmid into *E. coli* TOP10 yielded transformants resistant to β-lactams including carbapenems

Phenotypical characterization of OXA-517 β-lactamase. Comparison of antibiotic susceptibility profiles conferred by OXA-48, OXA-163, and OXA-517 was performed by cloning the corresponding genes into pCR-Blunt II-Topo kit (Invitrogen) and expressing them in *E. coli* TOP10 (Table 1). Unlike pTOPO-OXA-48, pTOPO-OXA-163 and pTOPO-OXA-517 conferred similar resistance phenotype for ceftazidime (MIC 16 mg/L and 4 mg/l respectively), conferred decreased susceptibility to aztreonam and an increased susceptibility to temocilline (32 mg/L). At the same time, pTOPO-OXA-517 conferred decreased susceptibility to carbapenems similarly to pTOPO-OXA-48, with MIC value for imipenem of 0.38 mg/ml (Table 1).

Biochemical properties of OXA-517. Steady-state kinetics parameters were determined in order to compare the catalytic properties of OXA-517 with those of OXA-48 and OXA-163. The catalytic efficiency of OXA-517 for ceftazidime was overall close to that obtained for OXA-163 (2 mM⁻¹s⁻¹ and 3 mM⁻¹s⁻¹ respectively). Conversely, OXA-48 was not capable of hydrolyzing ceftazidime (Table 2). Unlike OXA-163, OXA-517 had a catalytic activity for imipenem similar to that of OXA-48, and despite the fact that the hydrolysis rate of OXA-517 for imipenem was ~25-fold lower than that of OXA-48, it was ~250-fold higher in comparison with OXA-163 (Table 2). These results are in agreement with the MIC values given above. OXA-517 had poor affinity for temocillin, with *K_m* ~15-fold higher than OXA-48. Another notable difference with OXA-48 and also with OXA-163, concerned the behavior of OXA-517 with penicillins. When OXA-517 was used undiluted we observed no hydrolysis of different penicillins. Not only ampicillin and oxacillin, but also piperacillin, amoxicillin and bencilpenicillin were not hydrolyzed with the undiluted enzyme. The addition of NaHCO₃ as a source of CO₂ did not change the profile of hydrolysis of these penicillins.

Table 2. Kinetic parameters of OXA-48, OXA-517 and OXA-163, with representative β -lactam substrates.

Substrate	K_M (μ M)			k_{cat} (s^{-1})			k_{cat}/K_M ($mM^{-1}s^{-1}$)		
	OXA-48	OXA-163	OXA-517	OXA-48	OXA-163	OXA-517	OXA-48	OXA-163	OXA-517
Ampicillin	395	315	226	955	23	58	2418	70	256
Oxacillin	95	90	48	130	34	30	1368	370	629
Temocillin	45	N.H.	729	0.30	N.H.	0.24	7	N.D.	0.32
Cefalotin	195	10	>1000	44	3	>59	226	300	38
Cefoxitin	>200	N.D.	>1000	>0.05	N.D.	>6	0.26	N.D.	0.38
Ceftazidime	N.H.	>1000	>1000	N.H.	8	>20	N.D.	3	2
Cefotaxime	>900	45	>1000	>9	10	>22	10	230	16
Cefepime	>550	N.D.	>1000	>0.60	N.D.	>18	1.1	N.D.	11
Imipenem	13	520	411	5	0.03	6	369	0.06	14
Meropenem	11	>2000	>1000	0.07	0.10	>0.9	6	0.03	0.89
Ertapenem	100	130	>1000	0.13	0.05	>2.6	1.3	0.30	0.63
Aztreonam	N.H.	230	319	N.H.	0.50	1	N.D.	2	3.1

^aCLA, clavulanic acid (2 mg/L)^bTZB, tazobactam (4 mg/L)^c (4 mg/L)

The hydrolysis of penicillins was only achieved, when the enzyme was diluted at least 100 times (up to 100 000 times). With this dilution, it was possible to determine the steady-state parameters. OXA-517 presented a catalytic efficiency ~ 9 -fold lower than OXA-48 but ~ 4 -fold higher than OXA-163. These results correlate with the differences in enzymatic turnover, while the K_m values are similar for the three enzymes (Table 2). Unlike OXA-48, OXA-517 was capable of hydrolyzing aztreonam, with a catalytic efficiency similar to that of OXA-163 ($3.1\text{ mM}^{-1}\text{ s}^{-1}$ and $2.0\text{ mM}^{-1}\text{ s}^{-1}$ respectively).

Inhibition studied by determining the IC_{50} values, indicated that OXA-517 was more potently inhibited by clavulanic acid (0.014 mM) and tazobactam (0.002 mM) than OXA-48 (0.030 mM and 0.02 mM, respectively) (Table 3). However, the inhibition efficiency with NaCl was lower for OXA-517 than for OXA-48 (Table 3).

Crystal structure of OXA-517

Four OXA-517 protein molecules are present in the asymmetric unit of the crystal and they form two non-

crystallographic dimers related by pseudotranslation. Each dimer has a two-fold rotational symmetry, with the symmetry axis approximately parallel to the [110] direction. The electron density maps are of good quality with all four polypeptide chains clearly defined. Each subunit possesses a two-domain structure with a helix bundle domain and an α/β domain with a central seven-stranded antiparallel β -sheet, as described for OXA-48 (Docquier et al, 2009) (Fig. 3). Compared to OXA-48, OXA-517 carries an Ile215-Glu216 deletion coupled with an Arg214Lys mutation in the functionally important $\beta 5$ - $\beta 6$ loop. In OXA-517 the loop is comprised of residues Thr213, Lys214, Pro217 and Lys218 (residue numbering corresponding to OXA-48).

Table 3. Fifty percent inhibitory concentration (IC_{50}) of clavulanic acid, tazobactam and NaCl for β -lactamases OXA-48 and OXA-517.

Inhibitor	IC_{50} (mM)	
	OXA-48	OXA-517
Clavulanic acid	0.030	0.014
Tazobactam	0.020	0.002
NaCl	35	400

All the backbone atoms within the loop are visible in the electron density map but are not well ordered as indicated by their high temperature factors (Fig. 3, Fig. 4). The side chain of Lys214 is not visible in the electron density in any of the molecules. The peptide bond between Lys214 and Pro217 has a *cis* configuration as observed in OXA-48. A neighboring loop (residues 241-246), connecting the $\beta 7$ strand with helix $\alpha 10$, shows a similarly high mobility, with high average B values (Fig. 3).

The four protein molecules are nearly identical with rmsd values between 0.05 and 0.32 Å for their C α superpositions. The largest deviations between the subunits are visible at the N-terminus and within the loop between helices $\alpha 3$ and $\alpha 4$, which participates in crystal-lattice interactions.

Dimer interface. The OXA-517 dimer interface is the same as in OXA-48. A sulfate ion is bound between two Arg206 residues contributed by the dimer-forming subunits (Fig. 5A, B). Refining the sulfate ions at full occupancy resulted in negative difference density peaks (Fig. 5C) and poor electron density fit criteria. Refinement at 0.5 occupancy gives good fit to the electron density but results in the appearance of a localized positive difference density peak in the position right in the middle between the two arginine residues (Fig 5D). Modeling a half-occupancy chloride ion in this position resulted in clear electron density maps and realistic temperature factors for both partially occupied anions (Fig. 5B). In the structures of OXA-48 (PDB id 4s2p) and OXA-163 (4s2l) that we used for comparisons, a water molecule was modeled in this position. However, in both cases the water had unrealistically low temperature factors and we noted positive difference density peaks. Refinement of a chloride ion gave good electron density fit with no peaks in the difference map and B factors similar to those of the environment. We

conclude that in those two structures, a chloride ion is also bound at each dimer interface. The Arg206 residue that interacts with the bound anions originates at the amino end of the $\beta 5$ strand, opposite to the $\beta 5$ - $\beta 6$ loop. **The active site.** The active site is located at the interface of the two domains, between helices $\alpha 3$, $\alpha 5$ and $\alpha 6$, and the $\beta 5$ strand. All the active site residues are well defined in the electron density map, including the carboxylated Lys73 (Kcx) residue. The O γ atom of the catalytically important Ser70 nucleophile exhibits considerably higher B factor than its surroundings, except in chain B. The full hydrogen bond network within the active site is shown in Fig. 5. The O γ atom of Ser70 interacts with Kcx73, with the hydroxyl group of Ser118 and with a water molecule bound between the amide NH groups of Ser70 and Tyr211 (the oxyanion hole). This water molecule also interacts with the main-chain carbonyl of Tyr211. A sulfate ion is bound in the active site in all the four protein subunits and forms an ionic interaction with Arg250.

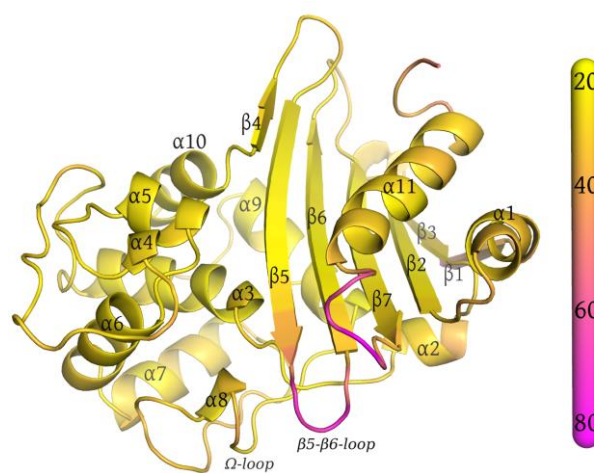


Figure 3. Cartoon model of a single subunit of OXA-517. The secondary structure elements are labeled according to (Docquier et al). The model is colored by the B factor of the C α atoms and the bar shows the color range in Å².

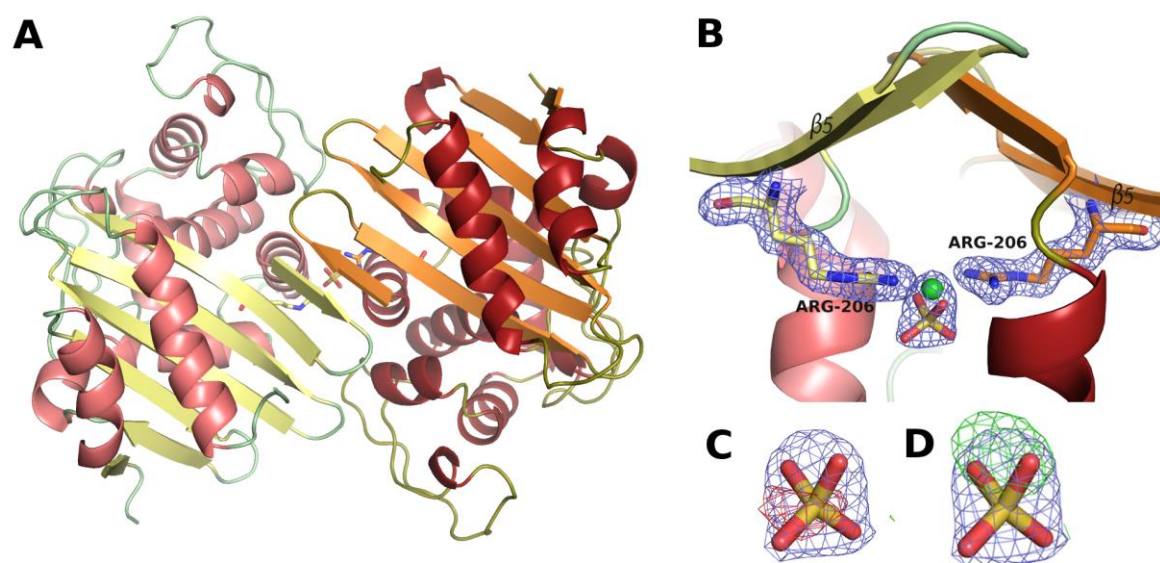


Figure 4. (A) An OXA-517 dimer shown as a transparent cartoon model with two Arg206 residues and the bound sulfate ion shown as sticks. (B) A zoom-in view on the dimer interface with the partially occupied chloride (green sphere) and sulfate (red and yellow sticks) ions. Two equivalent arginine residues from the complementary subunits interact with the ions. This view is rotated 90° upwards compared to A. (C) The sulfate ion when modeled at full occupancy, in the 2mFo-DFc (blue, contoured at 1.5 σ) and mFo-DFc (+/-3.0 σ , green/red) electron density maps, showing negative difference density. (D) The sulfate modeled at 0.5 occupancy with the electron density maps contoured at the same level as in C. A positive difference density peak is well visible, finally modeled as partial-occupancy Cl⁻ anion (green sphere in B).

The orientation of this anion is different in each of the subunits. The sulfate ion forms hydrogen bonds with the Oy1 atom of Thr209 and water in all chains, and with the N ζ atom of Lys208 in chains A, B and C. Only one water position within the active site is conserved between the four protein subunits.

Similarly, as in OXA-48, the walls of the active site cavity are lined by the Leu158 side chain and by residues in the β 5 strand, Tyr211-Ser212-Thr213. The groove ends with a hydrophilic cap formed by Oy1_Thr213, N_Thr213, Oy_Ser212 and O_Leu158.

Comparison with OXA-48 and OXA-163

The molecular structure of OXA-517 aligns well with that of OXA-48 (PDB id 4s2p) with an average rmsd of 0.50 Å for 238 superposed C α

atoms, and with OXA-163 (4s2l) with an average rmsd of 0.28 Å for 237 C α atoms (Fig. 6A). The total number of C α atoms in the aligned chains was equal to 244 for OXA-517, 238 for OXA-163 and 243 for OXA-48. The largest differences between these structures are found in the β 5- β 6 loop, where each protein has a slightly different sequence. The seven-residue loop (with the sequence 212-STRIEPK) in OXA-48 is replaced by a shorter sequence (212-STKPK) in OXA-517, and shorter yet in OXA-163 (212-DTK). In OXA-48, Arg214 forms a salt bridge with Asp159. This arginine is replaced by Lys214 in OXA-517 but a similar interaction is not observed in the structure of OXA-517. The side chain of Lys214 itself is not visible in the electron density but the loop is considerably shorter and there is a 3.8 Å distance

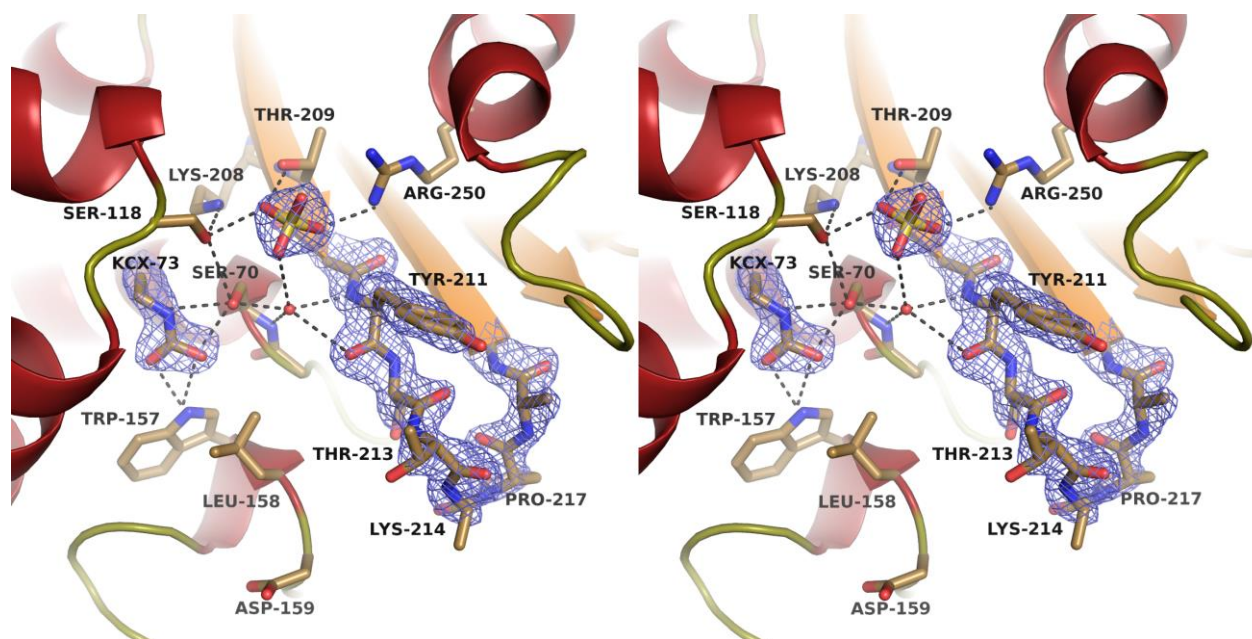


Figure 5. A stereo view of the active site of OXA-517 with residues implicated in substrate binding and the hydrolysis reaction shown as sticks. The only water molecule (small red sphere) in the active site that is preserved in all four protein subunits in the crystal is bound in the oxanion hole. 2mFo-DFc electron density (contoured at 1 σ) is shown for the carboxylated lysine residue (Kcx73), the sulfate ion and the residues in loop β 5- β 6.

between the C α atoms of Arg214 and Lys214 in the superposed structures of OXA-48 and OXA-517. An interaction between Lys214 and Asp159 would not be possible without significant conformational changes in either the β 5- β 6 or the Ω loop. A common feature of the β 5- β 6 loop in OXA-48 and OXA-517 is a *cis* peptide bond preceding Pro217 and a similar backbone conformation of this residue even though it has a different position and orientation (Fig. 6B). The side chain of Thr213 has a different orientation in the two structures with the Oy1 atom in OXA-517 pointing more ‘down’ toward the bottom of the binding cleft (Fig. 6 C,D). The points where the loop main chain atoms diverge most significantly lies between the C α atoms of Thr213 and Lys218; before and after those pivots the backbone atoms align well with each other. This is in contrast to the structure of OXA-163 where the main chain atoms on the side of the β 5 strand only converge with the

other two other structures at the N atom of Asp212 (Fig. 6B, E). In OXA-517 and OXA-48 the carbonyl O atom of Ser212 forms a hydrogen bond with N_Ile219. The Asp212-Thr213 peptide bond in OXA-163 has a different conformation and there is no hydrogen bond with N_Ile219. The side chain of Asp212 in this protein folds towards the loop and interacts with N_Lys218. Also, the side chain of Tyr211 has a different position in OXA-163 compared with the other structures but the electron density for this residue is weak. The relevant details of these comparisons are illustrated in Fig. 6B-E.

Docking of ceftazidime. The docked ceftazidime molecule fits well into the active site cleft with the aminothiazole ring placed between Thr213 from the β 5- β 6 loop and Leu158 from the Ω loop (Fig. 6F). The ring appears to fit perfectly into the small pocket surrounded by these two side chains, with

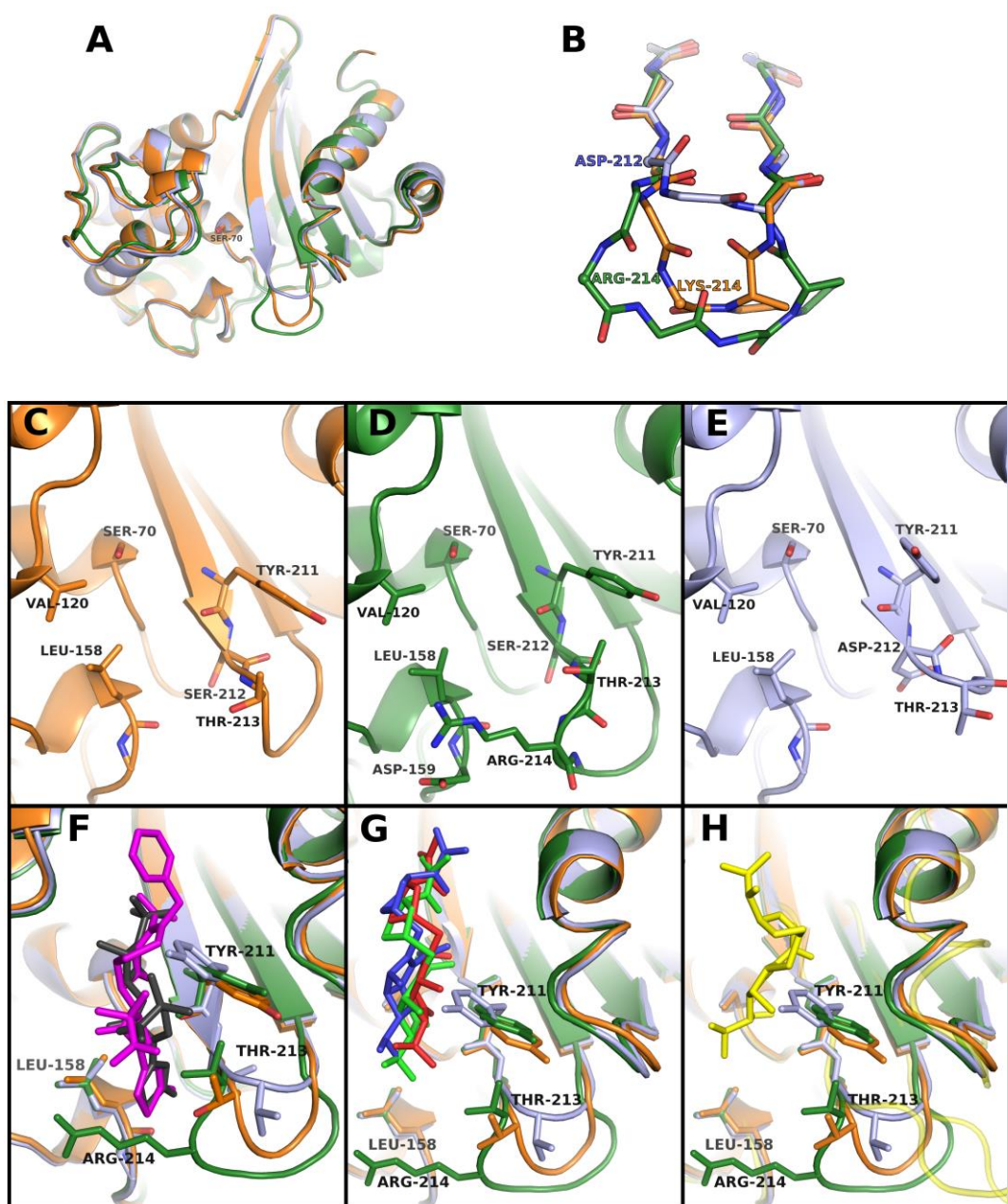


Figure 6. (A) Alignment of the structures of OXA-517 (orange), OXA-48 (green) and OXA-163 (gray) showing variation in the length of the β 5- β 6 loop. (B) A close-up view of the backbone conformation of the loop with visible *cis*-peptide bonds preceding Pro217. Also visible is the difference in backbone conformation between OXA-163 (gray) and the other structures at Asp212. The distance between the C α atoms of Arg214 (OXA-48) and Lys214 (OXA-517) is 3.8 Å. (C)-(E) Detailed views of the binding site in OXA-517 (C), OXA-48 (D) and OXA-163 (E) with key residues rendered as sticks. All three figures have the same orientation. (F) Ceftazidime (purple sticks) and aztreonam (dark gray) molecules docked into the active site of OXA-517 (orange), with aligned structures of OXA-48 (PDB id 4s2p, green) and OXA-163 (4s2l, light blue) superposed. (G) Results of docking of meropenem into the binding sites of OXA-517 (red), OXA-48 (green) and OXA-163 (blue). (H) Alignment of OXA-517 (orange) with the structure of OXA-13 (PDB id 1h8y, transparent yellow cartoon) complex with hydrolyzed meropenem (yellow sticks), showing the different position of the hydroxyethyl group of the ligand. Orientation as in G.

the amino group possibly forming hydrogen bonds with O_{Leu158} and O_γ-Ser212. By querying the PDB we found three structures of related proteins with hydrolyzed ceftazidime covalently attached to the active-site serine residue. They are: OXA-160, of type OXA-24/40, V160D mutant (PDB id 4x56, rmsd 1.23 Å for 224 Cα atoms aligned with OXA-517), OXA-225 K82D, of type OXA-23, (4x55, rmsd 1.27 Å for 234 aligned Cα atoms) (Mitchell et al, 2015), and a BlaR1 sensor domain from *Staphylococcus aureus* (1xkz, rmsd 1.41 Å for 226 aligned Cα atoms) (Birck et al, 2014). In each of these structures, the exact conformation of the bound ligand is different, but the consensus is that the aminothiazole ring is pointing ‘down’ to form close contacts with the protein, and the imino side chain carrying the carboxylate group pointing ‘up’ toward solvent. In all of these complexes the amide nitrogen of the R1 side chain of ceftazidime forms a hydrogen bond with the structural equivalent of O_{Tyr211}. This is in agreement with the conformation of ceftazidime generated in the present OXA-517 docking experiment. However, in the covalent acyl-enzyme complexes, the aminothiazole ring is buried more deeply inside the protein binding cavity and is at 90° angle compared to the aminothiazole ring in the docked conformation.

Fig. 6F shows the docked ceftazidime in the active site of OXA-517 and the structures of OXA-48 and OXA-163 aligned on it. We also docked a ceftazidime molecule into the active sites of OXA-48 and OXA-163, obtaining similar results in each case (data not shown). However, in the case of OXA-48 the antibiotic molecule cannot be fitted so deeply into the cavity due to clashes between the aminothiazole ring and the side chains of Thr213 and Arg214. The binding site cleft of OXA-163 is even larger than in OXA-517 and the ceftazidime molecule fits there very well without any problems.

Docking of aztreonam

The results of aztreonam docking are shown in Fig. 6F. The modeled antibiotic conformation is similar for all three (OXA-517, OXA-163, OXA-48) enzymes and is also very similar to that calculated for ceftazidime. We found only one similar crystal structure with covalently bound hydrolyzed aztreonam: OXA-160, V130D mutant (PDB id 4x53, rmsd 1.22 Å for 222 aligned Cα atoms). In the acyl-enzyme complex the aminothiazole ring of the ligand is buried deeply in the binding pocket and it seems to occupy the space of the Ω loop which in this structure is disordered and not visible in the electron density.

Docking of meropenem. To investigate the structural basis of carbapenemase activity, we docked a meropenem molecule into the active sites of OXA-517, OXA-48, and OXA-163. The simulated lowest energy binding modes are similar for all three proteins but in each case the ligand has a slightly different orientation (Fig. 6G). The β-lactam carbonyl is bound in the oxyanion hole, forming hydrogen bonds with N_{Tyr211} and N_{Ser70}.

Our search of the PDB for structurally similar proteins that contained a covalently bound hydrolyzed carbapenem compound returned the following hits: OXA-13 with hydrolyzed meropenem (PDB id 1h8y) (Pernot et al, 2001), OXA-23 with hydrolyzed meropenem (4jf4) (Smith et al, 2013), OXA-24/40 K84D mutant with doripenem (3pae) (Schneider et al, 2011), OXA-51 I129L/K83D double mutant with covalently bound doripenem (5l2f) (June et al, 2016), Bpu1 β-lactamase with doripenem (5ctn) (Toth et al, 2015), meropenem- and imipenem-acylated BlaR1 sensor domain from *Staphylococcus aureus* (3q82 and 3q81) (Borbulevych et al, 2011). The most structurally similar complex is that of OXA-13 with hydrolyzed meropenem. The rmsd between that structure and OXA-517 is 0.99 Å for 223 aligned

residues. Fig. 6H shows the alignment of that complex with OXA-517, OXA-163, and OXA-48. Overall, the docked ligand conformations are similar to that observed in the structure of the OXA-13 acyl-enzyme complex. The largest differences are seen for the hydroxyethyl group of the ligand.

Discussion

In this study, we have characterized a novel OXA-48 variant, OXA-517, recovered from a clinical isolate of *K. pneumoniae* from Belgium. OXA-517 hydrolyzes both expanded-spectrum cephalosporins and carbapenems. OXA-163 and OXA-405 have a four-residue deletion in the region from Tyr-211 to Pro-217 in the β 5- β 6 loop and hydrolyze expanded-spectrum cephalosporins but not carbapenems (Poirel et al, 2011; Dortet et al, 2015), whereas the OXA-48-like variants that possess carbapenemase activity have only substitutions of some residues in the β 5- β 6 loop region but no deletions. It is worth noting that contrary to what was observed for the OXA-48 variants OXA-163 and OXA-405, OXA-517 displays a deletion of only two amino acid residues in the β 5- β 6 loop and this is sufficient to confer resistance to expanded-spectrum cephalosporins as well as carbapenems. This is yet another fact that supports the hypothesis that substrate specificity of class D oxacillinases with respect to CHDL resides, at least in part, in the extent and amino acid composition of loop β 5- β 6 (Docquier et al, 2009; Stojanoski et al, 2015 ; Meziane-Cherif et al, 2016; De Luca et al, 2011).

Recently, temocillin has been proposed as a marker for the detection of OXA-48-like carbapenemase-producing *Enterobacteriaceae* (Huang et al, 2014). Indeed, a diameter <12 mm for temocillin on disk diffusion antibiograms suggests the presence of OXA-48-producers with a high specificity (90%) (Huang et al, 2014). OXA-517 producing *K. pneumoniae* was not able to growth on the OXA-48 side of

chromID® CARBA SMART (BioMérieux, Paris, France) even though the diameter around temocillin was 12mm (data not shown), corresponding to a MIC value of 192 mg/l. For this reason, our results seem to challenge the role of temocillin as a screening marker of OXA-48-like enzymes with a true carbapenemase activity.

The *bla*_{OXA-517} gene was identified on a plasmid with the backbone similar to the IncL *bla*_{OXA-48}-bearing plasmid (pOXA-48) (Poirel et al, 2012), except for a deletion of ca. 31 kb including *ssb* gene, *mobC* and *mobA* genes, *nikB* and *nikA* genes, all Locus *Tra* and part of *excA* gene. In the pOXA-517 plasmid, this region was replaced by the insertion sequence IS1R. Since the deleted region includes several genes involved in the mobilization and conjugative process of plasmids (Potron et al, 2014), we assume that this genetic rearrangement has led to the abrogation of the conjugative properties of the pOXA-517 plasmid as compared to pOXA-48 and, indeed, pOXA-517 was not found to be self-conjugative. The efficient spread of the plasmid encoding the OXA-48 carbapenemase has been recently attributed to its de-repressed transfer properties (Potron et al, 2014). As a consequence, we might hypothesize that the genetic rearrangement observed in the pOXA-517 plasmid could be deleterious for the dissemination of OXA-517 compared to OXA-48.

Considering the ability of OXA-517 to hydrolyze extended-spectrum cephalosporins, our findings are consistent with the conclusions drawn for OXA-163 that a larger active site cavity allows for the binding and hydrolysis of the bulky ceftazidime substrate (Stojanoski et al, 2015). The expansion of the cavity results from shortening of the β 5- β 6 loop and the elimination of arginine at position 214. Arg214 in OXA-48 forms a salt bridge with Asp159 and occupies part of the binding cavity. Any movement of this residue to free up more space for substrate binding would involve breaking this ionic interaction. In OXA-517, position 214 is occupied by a lysine residue which does not form a similar contact. As the β 5- β 6 loop in OXA-517 is by two residues shorter, there is a 3.8 Å distance between the Ca atom positions of residue 214 in OXA-517 and in OXA-48, and a similar salt-bridge interaction between Lys214 and Asp159 would require a drastic conformational change. The

larger size of the cavity is also attributed to the length of the β 5- β 6 loop, which in OXA-517 and OXA-163 is shorter than in OXA-48.

On the other hand, the structural basis for carbapenemase activity is elusive. Our docking calculations with meropenem show similar results for the structures of OXA-163, OXA-48 and OXA-517. Some slight differences in the orientation of the modeled antibiotic molecule may be attributed to different conformations of Tyr211 (Fig. 6G). However, the side chain of this residue is not well defined in the electron density and exhibits high mobility, as indicated by the high temperature factors in the crystal structure of OXA-517, as well as in OXA-163 (pdb id 4s2l) and OXA-48 (4s2p). Therefore, the observed differences in the conformation of this residue are not likely to be significant. It is possible that the binding and acylation reaction for carbapenem antibiotics proceed in the same way for these three OXA enzymes, and only the deacylation step is considerably slower for OXA-163. The kinetic data presented for OXA-163 (Stojanoski et al, 2015) show that a reduced catalytic efficiency for carbapenems, compared to OXA-48, is mainly caused by a decrease in the turnover rate, with unchanged K_M . However, other literature data show that there is both an increase in the K_M value and a decrease in k_{cat} for the tested carbapenem compounds (Oueslati et al, 2015). The creation of an unusually stable carbapenem acyl-enzyme intermediate was described for OXA-10 (De Luca et al, 2011). It was also suggested that some orientations of the hydroxyethyl group may restrict access of the hydrolytic water molecule to the covalent intermediate, and thus prevent deacylation.

A possible explanation for the differences in carbapenemase activity of these three enzymes is that the β 5- β 6 loop undergoes conformational changes and moves closer to the substrate, thus influencing its conformation during the hydrolysis

reaction. Such a view has been already proposed by Docquier et al. (Docquier et al, 2009). In our OXA-517 crystal structure all the loop residues have high temperature factors, suggesting high conformational freedom of this structural element, in agreement with its proposed long-range movements. The *cis*-Pro217 residue that is present in both OXA-517 and OXA-48, but not in OXA-163, enforces a specific backbone conformation that cannot be adopted by any other residue. This may be an important aspect of the activity of these enzymes towards carbapenems. Indeed, the sequence comparisons presented by De Luca et al. (De Luca et al, 2011) revealed a conserved PxxG sequence motif (residues 217–220 in OXA-48 numbering) in all class D carbapenemases, but not in other OXA-type enzymes. Further structural and biochemical data would be needed to confirm the importance of the *cis*-Pro217 residue.

Our crystal structure of OXA-157 shows that the dimer interface is stabilized by a chloride or/and sulfate anions bound between two Arg206 residues from the neighboring subunits. In OXA-10, the structurally equivalent His203 residue forms part of a metal ion binding site (Paetzel et al, 2000). Interestingly, it has been shown that supplementation of the buffer with some transition metal ions shifts the equilibrium in solution towards the dimeric form of this enzyme, which in turn increases its activity. An attractive hypothesis suggests itself, that the evolutionary advantage of OXA-48-like proteins, compared to OXA-10, is that their dimer formation is independent of metal cations and is instead stabilized by the abundantly present Cl^- ions.

Materials and Methods

Bacterial strains. *Klebsiella pneumoniae* 1219 was identified with matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MALDI, Biotyper CA system, Bruker Daltonics, Billerica, MA, USA). *Escherichia coli* TOP10

(Invitrogen, Saint-Aubin, France) and *E. coli* BL21 (DE3) (Novagen, VWR International, Fontenay-sous-Bois, France) were used for cloning experiments. Azide-resistant *E. coli* J53 was used for conjugation assays. *K. pneumoniae* 11978 and *K. pneumoniae* 6299 were used as reference strains for OXA-48 and OXA-163 cloning experiments, respectively (Poirel et al, 2015; Aubert et al 2006; Poirel et al, 2011).

Antimicrobial agents, susceptibility testing, and microbiological techniques. Antimicrobial susceptibilities were determined by disk diffusion technique on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the EUCAST breakpoints, updated in 2016 (<http://www.eucast.org>). Minimal inhibitory concentration (MIC) values were determined using the Etest technique (BioMérieux, Paris, France) and broth microdilution (Sensititre, Thermofisher, Montigny le Bretonne, France). *K. pneumoniae* 1219 was plated in different chromogenic medias: chromID® ESBL (BioMérieux, Paris, France) and chromID® CARBA SMART (BioMérieux, Paris, France).

Biochemical and immunochromatographic detection of carbapenemase-producing *Enterobacteria*.

Carbapenemase activity was investigated using the Carba NP test (Normann et al 2012), RAPIDEC® CARBA NP (BioMérieux, Paris, France), CARBA™ Test (Bio-Rad) (Bernabeu et al, 2017) and BYG test (Bogaerts et al, 2016; Bogaerts et al, 2017), as previously described (Normann et al 2012; Bernabeu et al, 2017; Bogaerts et al, 2016; Bogaerts et al, 2017). The expression of carbapenemase was investigated using OXA-48 K-Set immunochromatographic assay (Coris BioConcept, Gembloux, Belgium) as recommended by the manufacturer.

PCR, cloning experiments, and DNA sequencing. Whole-cell DNA of *K. pneumoniae* 1219 and *K. pneumoniae* 11978 isolates were extracted using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France) and were used as templates for PCR with the following primers: preOXA-48A (5'-TATATTGCATTAAGCAAGGG-3') and OXA-48B (5'-AAAAGGATCCCTAGGGAATAATTTTTCCTGTTGAGCA-3')

in order to amplify *bla*_{OXA-517} and *bla*_{OXA-48} genes, respectively. The amplicons obtained were then cloned into the pCR®-Blunt TOPO® plasmid (Invitrogen, Illkirch, France), downstream of the

pLac promoter, in the same orientation, resulting in pTOPO-OXA-517 and pTOPO-OXA-48. The recombinant pTOPO-OXA plasmids were electroporated into *E. coli* TOP10 cells; the electroporants were plated on a TSA plate containing kanamycin (50 ug/ml). The *bla*_{OXA-517} gene fragment corresponding to the mature β -lactamase was cloned into the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois, France) using the PCR-generated fragment with primers INF-OXA-48Fw (5'-AAGGAGATATACATATGGTAGCAAAGGAATGGCAAG-3') and INF-OXA-48Rv (5'-GGTGGTGGTCTCGAAGGGAATAATTTTTCCTGTTGAG-3') and the NEBuilder® HiFiDNA Assembly Cloning Kit (New England BioLabs® Inc, United Kingdom), following the manufacturer's instructions. The recombinant plasmid pET41-OXA-517 was transformed into the chemically-competent *E. coli* strain BL21 (DE3).

Recombinant plasmids were extracted using the Qiagen miniprep kit and both strands of the inserts were sequenced using M13 primers for the pCR®-Blunt II-TOPO® plasmid (Invitrogen, Illkirch, France), and T7 primers for pET41b(+) (Novagen, VWR International, Fontenay-sous-Bois, France), with an automatic sequencer (ABI Prism 3100; Applied Biosystems). The nucleotide sequences were analyzed using software available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

Whole genome sequencing (WGS). Total DNA was extracted from colonies using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, US) following the manufacturer's instructions. The DNA concentration and purity were controlled by a Qubit® 2.0 Fluorometer using the dsDNA HS and/or BR assay kit (Life technologies, Carlsbad, CA, US). The DNA library was prepared using the Nextera XT-v3 kit (Illumina, San Diego, CA, US) according to the manufacturer's instructions and then run on MiSeq (Illumina) for generating paired-end 300-bp reads. De novo assembly was performed by CLC Genomics Workbench v 9.5.3 (Qiagen, Hilden, Germany) after quality trimming (Qs $\geq$ 20) with word size 34. The acquired antimicrobial resistance genes were identified by uploading the assembled genomes to the Resfinder server v2.1 (<http://cge.cbs.dtu.dk/services/ResFinder-2.1>) (Zankari et al, 2012). The Multi Locus Sequence Typing (MLST) and the identification of the different plasmids were also obtained by uploading the assembled genomes to the MLST 1.8 (Jorgensen et al, 2012) and PlasmidFinder 1.3 (Carattoli et al, 2014) servers, respectively, available at <https://cge.cbs.dtu.dk/services/>

Plasmid characterization and conjugation assay.

Plasmid DNA of the clinical isolates *K. pneumoniae* 1219 and *K. pneumoniae* 11978 were extracted using the Kieser method (Kieser et al, 1984). The Kieser extract from both *K. pneumoniae* isolates were used to transform *E. coli* TOP10 strain by electroporation. The electroporants were plated on a TSA plate containing 100 µg/ml ampicillin. Transformants were analyzed by PCR using the primers OXA-48A and OXA-48B (5'-TTGGTGGCATCGATTATCGG-3' and 5'-GCCATCACAAGAAGTGCTC-3', respectively). From the transformants harboring the *bla*_{OXA-517} gene, plasmid DNA extraction was done using the Kieser method and subsequently analyzed on 0.7% agarose gel stained with ethidium bromide. Plasmids of ca. 154, 66, 48, and 7 kb of *E. coli* NCTC 50192 were used as plasmid size markers (Dortet et al, 2015).

To perform the quantitative filter mating-out assay, the clinical isolates *K. pneumoniae* 1219 and *K. pneumoniae* 11978 were used as donors. The donors and the recipient *E. coli* J53 were each grown overnight in brain heart infusion (BHI) (BioMérieux, Paris, France) broth supplemented with 100 µg/ml ampicillin for plasmid maintenance in donor cells. A 0.25 ml sample of donor culture was mixed with 4.75 ml BHI broth and incubated at 37°C for 5 h without shaking. Recipient cultures of *E. coli* J53 grown overnight were diluted 1:50 in BHI broth and incubated at 37°C for 5 h with shaking. After incubation, 0.25 ml of the donor culture was gently mixed with 2.5 ml of the recipient culture, and 200 µl of this mating mix was filtered through a 0.45-µm filter (Millipore). Filters were incubated on prewarmed plates at 37°C for 2 h. The mating assays were terminated by placing the filters in 4 ml of an ice-cold 0.9% NaCl solution, followed by vigorous agitation for

30 s. The number of transconjugants per donor cell was determined by plating dilutions of the mating mixture onto plates containing antibiotics. Strain donor cells were selected with 100µg /ml ampicillin. Transconjugants were selected with 100µg/ ml ampicillin and 100µg/ ml azide. Transfer frequencies were calculated by dividing the number of transconjugants by the number of donor cells.

β-Lactamase purification. An overnight culture of *E. coli* strain BL21 (DE3) harboring pET41b-OXA-517 was used to inoculate 2 L of LB broth containing 50 mg/L kanamycin. The bacteria were grown at 37°C until the culture reached an OD₆₀₀ of 0.6. Expression of OXA-517 was induced overnight at 25°C with 0.2 mM IPTG, as previously described (Dabos et al, 2018). Cultures were centrifuged at 6000 g for 15 min and the pellets were resuspended in 10 mL of Buffer A (20 mM phosphate buffer, 175 mM K₂SO₄, 40 mM imidazole, pH 7.4). The cells were disrupted by sonication and bacterial debris was removed by two consecutive 1 h centrifugation steps at 4°C: at 10000 g, and at 48000 g. OXA-517 was purified in one step pseudo-affinity chromatography using an NTA-Ni column (GE Healthcare, Freiburg, Germany) (Dabos et al, 2018). Protein purity was estimated by SDS-PAGE, pure fractions were pooled and dialyzed against 20 mM Hepes, 50 mM K₂SO₄ buffer (pH 7.0) and concentrated using Vivaspins® columns (GE Healthcare, Freiburg, Germany). Protein concentration was determined by Bradford Protein assay (Bio-Rad, Marnes-La-Coquette, France) (Bradford, 1976).

Steady-state kinetic parameters. Kinetic parameters of purified OXA-517 were determined at 30°C in 100 mM sodium phosphate buffer (pH 7.0). The *k*_{cat} and *K*_m values were

determined by analyzing hydrolysis of β -lactams under initial-rate conditions with an ULTROSPEC 2000 model UV spectrophotometer (Amersham Pharmacia Biotech) using the Eadie–Hoffstee linearization of the Michaelis–Menten equation, as previously described (Naas et al, 1998). The β -lactams were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Comparative activation of OXA-517 by CO₂ was conducted by monitoring the rate of hydrolysis of penicillins in the same buffer supplemented with 50 mM sodium hydrogen carbonate (NaHCO₃) as the source of CO₂ (Meziane-Cherif et al, 2016).

Crystallization, X-ray data collection, structure refinement and analysis.

A 27 mg/ml protein solution was used for crystallization screening with the application of the NeXtal crystallization kit (Qiagen). Single crystals of OXA-517 grew in 0.1 M MES pH 6.5 and 20% (w/v) PEG10000. The diffraction data (Table 4) were collected at the SOLEIL PROXIMA II beamline equipped with an Eiger detector. The images were processed and scaled with the XDS package (Kabsch, 2010; Krug et al, 2012) producing intensities merged in the *P2*₁ space group as output. The intensities were then converted to structure factor amplitudes with *Truncate* (French & Wilson, 1978) from the *CCP4* program suite (Winn et al, 2011). An inspection of the Patterson map, calculated using the FFT algorithm implemented in *fftbig* (Ten Eyck, 1973) from the *CCP4* package, revealed a large non-origin peak at 0.5, 0, 0.5, indicating translational pseudosymmetry. The structure was solved by molecular replacement using *Phaser* (McCoy et al, 2007) and taking advantage of the detected pseudotranslation. The starting model was a single subunit of OXA-48 (PDB id 4s2p). The calculations gave a single solution with a Z-score of 15.4 for the translation function and a final LLG of 2405. No twinning was found by the *Phaser* twin detection algorithm (Read et al, 2013), which corrects for translational NCS. An alternative solution, shifted by 0.25, 0, 0.25, was also tested but it gave significantly higher R factors. The *Achesym* server (Kowiel et al, 2014) was used to standardize the placement of the model inside the unit cell. Maximum-likelihood refinement was carried out in

Table 4. X-ray data collection and structure refinement statistics

<i>Data collection and processing</i>	
Wavelength [Å]	0.98010
Resolution [Å]	46.37–1.86 (1.91–1.86)*
Space group	<i>P2</i> ₁
Cell parameters [Å, °]	50.05/125.74/83.03/94.6
R _{meas} [%]	15.6 (125.3)
<I/σI>	6.57 (1.03)
CC _{1/2} [%]	99.5 (34.7)
Completeness [%]	98.5 (91.9)
No. of unique reflections	84532 (13554)
Redundancy	3.3 (3.0)
<i>Refinement</i>	
R _{work} /R _{free} [%]	17.0/21.9
Rmsd bond lengths [Å]	0.012
Rmsd bond angles [°]	1.37
No. of protein molecules in ASU	4
No. of modeled amino acid residues	979
No. of all/protein/water/other atoms	8981/8072/738/185
Wilson B factor [Å ²]	24.2
Average B factor [Å ²] protein/water/other solvent/all	30.9/39.0/43.0/31.6
Ramachandran favored/allowed [%]	98/2
PDB code	6hb8

Refmac5 (Murshudov et al, 2011), while model rebuilding and analysis was done in *Coot* (Emsley et al, 2010). For validation of the final model we used *Molprobit* (Chen et al, 2009) and the PDB validation server (Berman et al, 2000). The SSM algorithm implemented in *Coot* was used for 3D superpositions of

different models. Figures were generated with *Pymol* (DeLano, 2014). For comparisons of our structure with other models, we downloaded their coordinates and, when available, the corresponding electron density maps from the Uppsala Electron Density Server (EDS) (Kleywegt et al, 2004). For a few PDB models, we applied some minor adjustments to their coordinates to improve the electron density fit or stereochemical parameters. We used the *CCP4* package for conversions between file formats and *Truncate* to convert the intensities to structure factor amplitudes, if necessary.

Docking calculations were performed with *Autodock Vina* (Trott & Olson, 2010). Ligand coordinates were generated by the Grade Web Server (Smart et al, 2011). Input files were first processed with *AutodockTools* (Morris et al, 2009) to generate polar hydrogen atoms, compute Geisterger charges and choose rotatable bonds for the ligand. The receptor was treated as a rigid body. Docking was performed using a Lamarckian genetic algorithm in a grid box of 20 x 30 x 30 Å encompassing all of the active site volume.

Nucleotide sequence and PDB accession numbers The nucleotide sequence of the *bla_{OXA-517}* gene has been submitted to the EMBL/Genbank nucleotide sequence database, under the accession number KU878974. The nucleotide sequence of plasmid pOXA-517 has been submitted also, under the accession number KY200950. The Whole Genome Shotgun of Kp1219 has been deposited at the DDBJ/ENA/GenBank under the accession code NXNM000000000. The version described in this paper is version NXNM01000000. Atomic coordinates and structure factors for the crystal structure of OXA-517 have been deposited with the Protein Data Bank (PDB) with the accession number 6HB8.

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Conflict of interest

None to declare.

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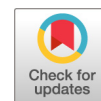
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Genetic and Biochemical Characterization of OXA-519, a Novel OXA-48-Like β -Lactamase

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ABSTRACT A multidrug-resistant *Klebsiella pneumoniae* 1210 isolate with reduced carbapenem susceptibility revealed the presence of a novel plasmid-encoded *bla*_{OXA-48-like} gene, named *bla*_{OXA-519}. The 60.7-kb plasmid (pOXA-519) was similar to the IncL-OXA-48 prototypical plasmid except for a ca. 2-kb deletion due to an IS1R insertion. OXA-519 differed from OXA-48 by a Val120Leu substitution, which resulted in an overall reduced β -lactam-hydrolysis profile, except those for ertapenem and meropenem, which were increased. Thus, detection of OXA-519 producers using biochemical tests that monitor imipenem hydrolysis will be difficult.

KEYWORDS carbapenemase, mutant, steady-state kinetics, OXA-48 like, detection

Class D β -lactamases (DBLs), or OXA-type β -lactamases (OXAs), form a very diverse family of enzymes (1–3), the diversity of which is reflected at both genetic and biochemical levels. OXA-48, the main carbapenem-hydrolyzing class D β -lactamase (CHDL) encountered in *Enterobacteriaceae* in many countries around the Mediterranean area, was initially identified from a carbapenem-resistant *Klebsiella pneumoniae* isolate from Turkey in 2001 (4, 5). Although OXA-48 hydrolyzes penicillins at a high level and carbapenems at a low level, it shows very weak or no activity against expanded-spectrum cephalosporins (6). Along with the spread of OXA-48, several variants have been reported that differ from OXA-48 by up to 5 amino acid substitutions or deletions (<http://bldb.eu/BLDB.php?class=D#OXA>) (7). The aim of this study was to characterize OXA-519, a novel OXA-48-like β -lactamase detected in a clinical *K. pneumoniae* isolate recovered in Belgium in 2015.

K. pneumoniae 1210 was recovered from a urine sample of an 87-year-old patient who had not been hospitalized in the last 12 months. The urine displayed hyperleukocyturia, and the culture grew $>10^5$ CFU/ml of *K. pneumoniae*, which was identified using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MALDI Biotyper; Bruker Daltonics, Illkirch, France). Disk diffusion antibiotic susceptibility testing was done and interpreted according to the EUCAST guidelines (<http://www.eucast.org>). The isolate *K. pneumoniae* 1210 was resistant to penicillins and expanded-spectrum cephalosporins, was intermediate to ertapenem, and

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TABLE 1 MICs of β -lactams for *K. pneumoniae* and *E. coli* strains^a

Antibiotic	MIC (mg/liter) against:									
	<i>K. pneumoniae</i> 1210	<i>E. coli</i> TOP10				<i>E. coli</i> TOP10	<i>E. coli</i> HB4			
		pNOXA-519	pR-OXA-48	pR-OXA-519	pR-OXA-163		pR-OXA-48	pR-OXA-519	pR-OXA-163 ^c	pN-OXA-519
Amoxicillin	>256	>256	>256	>256	>256	2	>256	>256	>256	>256
Amoxicillin + CLA ^b	48	32	192	32	96	2	>256	32	>256	8
Cefotaxime	>32	0.047	0.094	0.047	3	0.06	8	0.75	128	1.5
Ceftazidime	48	0.125	0.19	0.125	16	0.12	1	0.5	256	1
Cefepime	16	0.023	0.047	0.023	0.5	0.023	12	0.75		1
Imipenem	0.25	0.25	0.38	0.25	0.25	0.25	>32	1.5	0.5	>32
Meropenem	0.75	0.19	0.047	0.19	0.023	0.016	>32	>32	4	>32
Ertapenem	2	0.125	0.047	0.125	0.032	0.003	>32	>32	32	>32
Temocillin	>1,024	>1,024	>1,024	>1,024	32	4	>1,024	>1,024	64	>1,024
Aztreonam	>256	0.047	0.047	0.047	2	0.047	0.5	0.38		0.5

^a*K. pneumoniae* 1210, *E. coli* TOP10 pTOPO OXA-48, *E. coli* TOP10 pTOPO-519, *E. coli* TOP 10 pTOPO-163, *E. coli* TOP10, *E. coli* HB4 pOXA-48, *E. coli* HB4 pOXA-519, and *E. coli* HB4.

^bCLA, clavulanic acid (2 mg/liter).

^cValues from Oueslati et al. (15).

remained susceptible to meropenem and imipenem (Table 1). The Carba NP test, β -Carba test (Bio-Rad, Marnes-La-Coquette, France), Bogaerts-Yunus-Glupczynski (BYG) Carba test, and Maldi-TOF Star-BL assay (Brucker Daltonics) were performed as previously described (9–12). Carba NP and BYG Carba tests gave negative test results while β -Carba and Star BL gave weak but reproducible positive results. OXA-48 K-SeT (Coris BioConcept, Gembloux, Belgium) and the NG Carba 5 (NG Biotech, Rennes, France) immunochromatographic assays revealed a positive OXA-48 band (13, 14). PCR experiments and subsequent sequencing, as previously described (15), revealed the presence of a novel *bla*_{OXA-48-like} gene, designated the *bla*_{OXA-519} gene, which showed a single nucleotide change resulting in a single amino acid Val to Leu substitution at position 120, according to DBL numbering (3, 16, 17).

The *bla*_{OXA-48}, *bla*_{OXA-163}, and *bla*_{OXA-519} genes were amplified using preOXA-48A (5'-TATATTGCATTAAGCAAGGG-3') and cloning-OXA-48B (5'-AAAAGGATCCCTAGGGAA TAATTTTTCTGTGTTGAGCA-3') primers and were subsequently cloned into the pCR-Blunt II-TOPO plasmid (Invitrogen, Illkirch, France), resulting in recombinant pR-OXA-48, pR-OXA-163, and pR-OXA-519 plasmids, respectively. OXA-519 conferred similar, but reduced resistance profiles on *Escherichia coli* TOP10 compared to those conferred by OXA-48, consisting of susceptibility to expanded-spectrum cephalosporins, resistance to temocillin and piperacillin-tazobactam, and decreased susceptibility to imipenem (MIC, 0.25 μ g/ μ l). OXA-519 presented slightly higher MICs for meropenem and ertapenem compared to those presented by OXA-48 (~4-fold and ~3-fold, respectively) (Table 1), and it was more inhibited by clavulanic acid compared to OXA-48 and OXA-163 (Table 1). In order to see whether these enzymes may lead to carbapenem resistance in bacterial hosts with impaired outer-membrane permeability, pR-OXA-48, pR-OXA-163, and pR-OXA-519 plasmids were transformed into *E. coli* HB4 lacking the major porins *OmpF* and *OmpC* (15). In *E. coli* HB4, all three enzymes presented increased MICs for all of the β -lactams tested, including the carbapenems. For OXA-519, MIC values increased by more than ~256-fold for ertapenem and meropenem, thus resulting in resistance to these two molecules, but only by 6-fold for imipenem (Table 1), resulting in a MIC of 1.5 μ g/ μ l, which is still in the susceptibility range.

The *bla*_{OXA-519} gene was cloned into the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois, France) using the PCR-generated fragment with primers INF-OXA-48Fw (5'-AAGGAGATATACATATGGTAGCAAAGGAATGGCAAG-3') and INF-OXA-48Rv (5'-GGTGGTGGTGCTCGAAGGAATAATTTTCTGTTGAG-3'), and the NEBuilder HiFi DNA assembly cloning kit (New England BioLabs Inc., United Kingdom), following the manufacturer's instructions. Recombinant plasmid pET41-OXA-519 was electroporated into *E. coli* strain BL21(DE3) and expressed at 25°C with 0.2 mM IPTG

TABLE 2 Steady-state kinetic parameters of β -lactamases OXA-48, OXA-519, and OXA-163

Substrate	Kinetic parameter ^a								
	K_m (μ M)			k_{cat} (s^{-1})			k_{cat}/K_m (mM^{-1}/s^{-1})		
	OXA-48	OXA-519	OXA-163	OXA-48	OXA-519	OXA-163	OXA-48	OXA-519	OXA-163
Ampicillin	395	776	315	955	131	23	2,418	169	70
Piperacillin	410	109	ND	75	36	ND	180	328	ND
Oxacillin	95	338	90	130	8	34	1368	23	370
Temocillin	45	>1,000	NH	0.30	>12	NH	6.6	8.8	ND
Cephalothin	195	>1,000	10	44	>13	3.0	226	3.5	300
Cefoxitin	>200	>1,000	ND	>0.05	>4	ND	0.26	1.7	ND
Ceftazidime	NH	373	>1,000	NH	0.02	8	ND	0.04	3
Cefotaxime	>900	>1,000	45	>9	>1.3	10	10	0.39	230
Cefepime	>550	>1,000	ND	>0.60	>1.3	ND	1.1	0.31	ND
Imipenem	13	982	520	4.8	2.1	0.03	369	2.1	0.06
Meropenem	11	358	>2,000	0.07	3.4	0.10	6.2	9.5	0.03
Ertapenem	100	83	130	0.13	1.1	0.05	1.3	13	0.30

^aNH, hydrolysis could not be detected with concentrations of substrate and enzymes up to 1,000 mM and 400 nM, respectively; ND, not determined.

(isopropyl- β -D-thiogalactopyranoside) as inducer. OXA-519 was purified by one-step pseudo-affinity chromatography, using a nitrilotriacetic acid (NTA)-nickel column (GE Healthcare, Freiburg, Germany). Kinetic parameters were determined as previously reported (15). The lowest catalytic efficiencies were observed for cephalosporins. Interestingly, and unlike for OXA-48, hydrolysis of ceftazidime could be detected for OXA-519, although at lower level than that for OXA-163. With respect to carbapenems, OXA-519 had an increased catalytic efficiency for ertapenem (10-fold) and meropenem (2-fold), but the k_{cat}/K_m value for imipenem was 176-fold smaller than that of OXA-48 (Table 1). Unlike OXA-48, which is not inhibited by clavulanic acid and is well inhibited by NaCl (50% inhibitory concentration [IC_{50}], 7 mM) (4), OXA-519 was significantly inhibited by clavulanic acid (IC_{50} , 83 μ M) and weakly inhibited by NaCl (IC_{50} , 200 mM). This inhibition profile is similar to that of OXA-163, which has IC_{50} values of 13.4 μ M and 270 mM for clavulanic acid and NaCl, respectively (4, 15).

An analysis of all OXA-48-like sequences available in the Beta-Lactamase DataBase (7) showed that position 120 is very conserved within this family. The mutated residue in position 120 is situated at the bottom of the binding site (data not shown), in the close vicinity of the active Ser70 and the β 5- β 6 loop, and thus it is likely involved in the β -lactamase activity of OXA-48 (6, 18, 19). In OXA-51, the chromosomal CHDL of *Acinetobacter baumannii* that hydrolyzes carbapenems less efficiently than other CHDLs, position 120 is occupied by Ile instead of Val (20). The carbon δ of this Ile would cause a steric clash with the hydroxyethyl group of carbapenems, leading to an increase in the K_m values of OXA-51 (8, 21). Likewise, the bulkier side chain of Leu120 in OXA-519, compared to that of Val120 in OXA-48, hampers the approach of β -lactam substrate, resulting in a decrease of the substrate affinity. This is in agreement with higher K_m values determined for OXA-519 compared with those for OXA-48 (Table 2).

The whole genome of *K. pneumoniae* 1210 was sequenced using Illumina technology, as previously reported (22). The genome was 5,549,801 bp in size, with a mean coverage of 57 $\times$. Multilocus sequence typing (MLST) of *K. pneumoniae* 1210, determined using MLST 1.8 software (23), revealed a novel ST, which was assigned the novel allelic profile ST2728 (1-1-211-1-1-1) by the *K. pneumoniae* MLST database (<http://bigsd.db.web.pasteur.fr>). This sequence type is a single-locus variant (SLV) of ST15 (1-1-1-1-1-1-1), a pandemic clone widely described in association with carbapenemases or ESBLs and sometimes involved in outbreaks (24–26). The acquired resistance genes were identified using ResFinder server v2.1 (<http://cge.cbs.dtu.dk/services/ResFinder-2.1>) (27). *K. pneumoniae* 1210 presented four β -lactamases genes, including the naturally occurring bla_{SHV-28} , the acquired bla_{OXA-1} , the $bla_{CTX-M-15}$ ESBL, and the $bla_{OXA-519}$ carbapenemase genes. The bla_{OXA-1} gene, the $aac(6')-Ib-cr$ gene conferring resistance to kanamycin, tobramycin, and amikacin and decreased susceptibility to fluoroquinolones (28), and the *catB4* gene conferring chloramphenicol resistance were

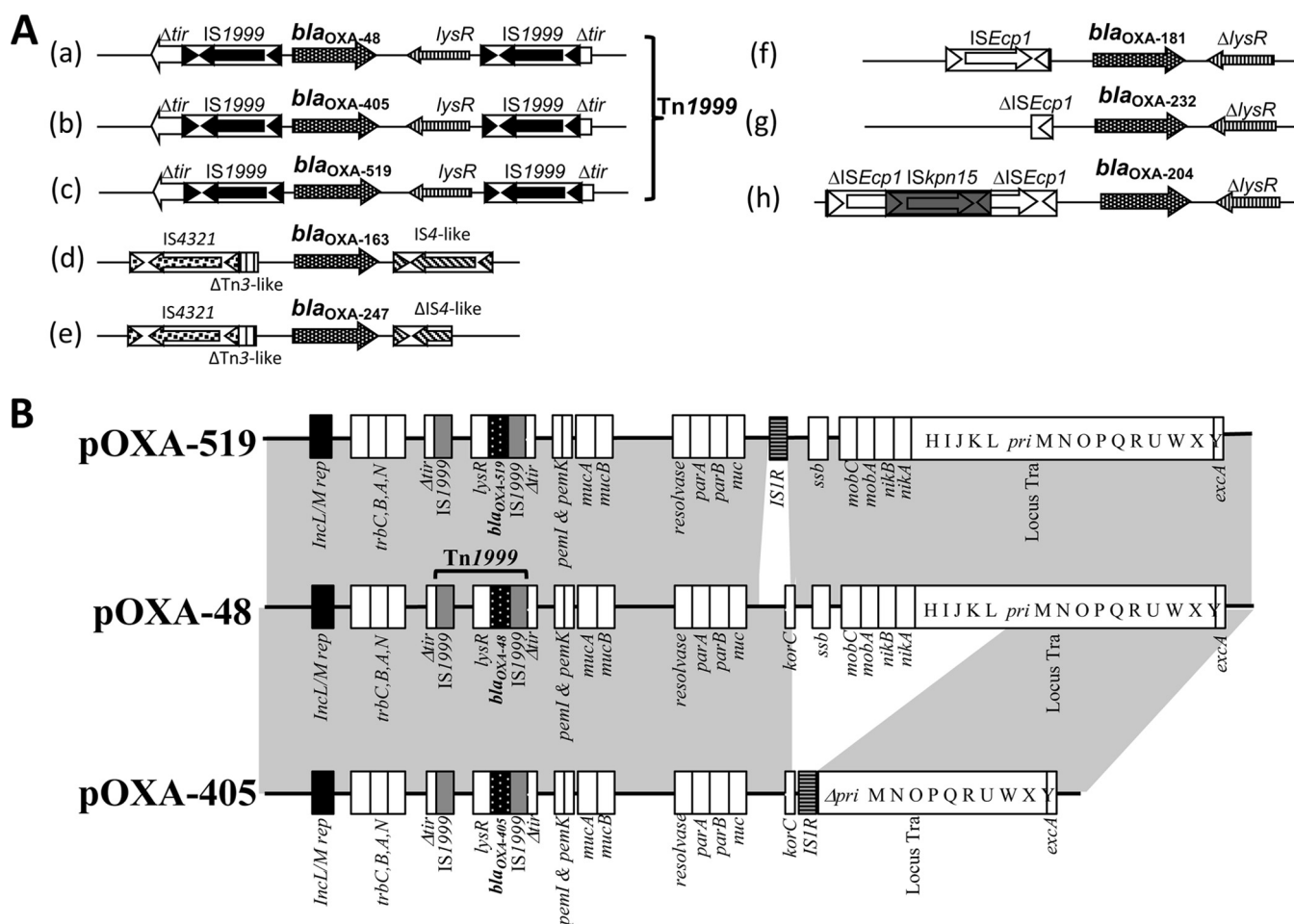


FIG 1 (A) Schematic representation of the genetic environment of bla_{OXA-48} -like β -lactamases. bla_{OXA-48} (a), $bla_{OXA-405}$ (b), $bla_{OXA-519}$ (c), $bla_{OXA-163}$ (d), $bla_{OXA-247}$ (e), $bla_{OXA-181}$ (f), $bla_{OXA-232}$ (g), and $bla_{OXA-204}$ (h) genes. The Tn1999 composite is formed by two copies of insertion sequence IS1999 bracketing a fragment containing the bla_{OXA-48} , $bla_{OXA-405}$, and $bla_{OXA-519}$ genes. (B) Major structural features of the plasmids pNOXA-519 from *K. pneumoniae* 1210 with the prototypical IncL bla_{OXA-48} plasmid (pOXA-48) (GenBank accession number [JN626286](https://www.ncbi.nlm.nih.gov/nuclink/JN626286)) and pOXA-405. Common structures are highlighted in gray.

part of a class 1 integron, as previously described (29). A *fosA*-like gene involved in the decreased susceptibility to fosfomycin in *K. pneumoniae* was also evidenced. In addition, GyrA topoisomerase exhibited several substitutions (S83F and D87A) that are known to confer high-level resistance to fluoroquinolones in Gram-negative rods (30). However, the *gyrB*, *parC*, and *parE* genes did not display any mutation in the quinolone resistance-determining region (QRDR) (data not shown). Finally, the *ompK35* gene coding for the OMPK35 porin was disrupted by the insertion sequence belonging to the IS1 family, while the *ompK36* gene was not altered.

The $bla_{OXA-519}$ gene was located on a Tn1999 composite transposon, unlike the $bla_{OXA-163}$ or $bla_{OXA-247}$ genes, which are associated with IS4361 and IS4 elements, and the $bla_{OXA-181}$, $bla_{OXA-204}$, and $bla_{OXA-232}$ genes, which are associated with the ISEcp1 element (Fig. 1A). Three different plasmid-replication origins belonging to the incompatibility groups, IncFIB, IncL, and IncFII(K) were identified using PlasmidFinder 1.3 (<https://cge.cbs.dtu.dk/services/>). The $bla_{OXA-519}$ gene was carried by an IncL-type plasmid of ca. 60 kb, similar to the prototypical pOXA-48 plasmid (GenBank accession number [JN626286](https://www.ncbi.nlm.nih.gov/nuclink/JN626286)) but differing by a 1,986-bp deletion encompassing the *korC* gene (31–33) (Fig. 1B). Conjugation assays, performed as previously described (34), revealed a high conjugation frequency of pN-OXA-519 ($1.05 \times 10^{-1} \pm 0.003$), which was similar to that of pOXA-48 ($1.13 \times 10^{-1} \pm 0.002$), suggesting that the deletion has no impact on the conjugation efficiency.

We report here a novel OXA-48-like β -lactamase, OXA-519, which presents reduced activity toward imipenem compared to that of OXA-48. It is important to stress that, even though OXA-519 has reduced imipenem-hydrolytic activity compared to that of OXA-48, it has increased meropenem and ertapenem catalytic efficiency, and when expressed in a porin-deficient strain, the MICs rose significantly in the resistance range. Of note, OXA-519 may spread silently, since conventional biochemical tests based on carbapenem hydrolysis failed to detect this variant. The mutation Val120Leu is located at the bottom of the active site cavity of the protein. The bulkier side chain of Leu in OXA-519 compared with that of Val in OXA-48 induces a decrease in substrate affinity. Considering the high transfer frequency of pOXA-519, which is similar to that of pOXA-48, the risk of dispersion of this gene in the gut of patients may result in high-level carbapenem resistance, even though the initial isolate may be susceptible.

Accession number(s). The nucleotide sequences of the *bla*_{OXA-519} gene and of its natural plasmid pOXA-519 have been submitted to the EMBL/GenBank nucleotide sequence database under the accession numbers [KX349732](#) and [KY215945](#), respectively. This whole-genome shotgun sequence of strain *K. pneumoniae* 1210 has been deposited at DDBJ/ENA/GenBank under the accession number [PCGD00000000](#). The version described in this paper is [PCGD01000000](#).

ACKNOWLEDGMENTS

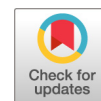
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We declare no competing interests.

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Genetic and Biochemical Characterization of OXA-535, a Distantly Related OXA-48-Like β -Lactamase

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ABSTRACT OXA-535 is a chromosome-encoded carbapenemase of *Shewanella bicestii* JAB-1 that shares only 91.3% amino acid sequence identity with OXA-48. Catalytic efficiencies are similar to those of OXA-48 for most β -lactams, except for ertapenem, where a 2,000-fold-higher efficiency was observed with OXA-535. OXA-535 and OXA-436, a plasmid-encoded variant of OXA-535 differing by three amino acids, form a novel cluster of distantly related OXA-48-like carbapenemases. Comparison of *bla*_{OXA-535} and *bla*_{OXA-436} genetic environments suggests that an *ISCR1* may be responsible for *bla*_{OXA-436} gene mobilization from the chromosome of *Shewanella* spp. to plasmids.

KEYWORDS OXA-48-like, carbapenemase, progenitor, OXA-48, *Shewanella*

OXA-48, a carbapenemase, has become a major public health threat because of its rapid spread worldwide (1–3). Along with this rapid dispersion, several OXA-48 variants have been reported (4–6). For a complete list of variants, see the β -lactamase database (<http://bldb.eu/BLDB.php?class=D#OXA>) (4). Analysis of the genetic context of *bla*_{OXA-48-like} genes in *Enterobacteriaceae* has shown their association with insertion sequences (IS1999, *ISEcp1*, and *IS4321*) and with various plasmids (7–10). *Shewanella* spp. constitute the reservoir of *bla*_{OXA-48-like} genes. So far, the gene variants *bla*_{OXA-48}, *bla*_{OXA-199}, *bla*_{OXA-204}, and *bla*_{OXA-416} genes have been reported in *Shewanella xiamensis*, although some researchers have identified *bla*_{OXA-48-like} genes in other *Shewanella* spp. (11–17). OXA-535, the naturally occurring oxacillinase of *Shewanella bicestii* JAB-1, share only 91.3% and 98.9% amino acid sequence identities with OXA-48 and the plasmid-borne OXA-436, respectively (5, 18) (Fig. 1A). The aim of this study was to characterize the biochemical properties of OXA-535 and investigate its genetic environment in *S. bicestii* JAB-1.

MIC values were determined as previously described (18). *Escherichia coli* TOP10 (Invitrogen, Saint-Aubin, France) expressing OXA-535 from the pTOPO plasmid was resistant to penicillins, including temocillin, and reduced susceptibility to carbapenems; whereas the expanded-spectrum cephalosporins remained in the susceptibility range, in a manner similar to that of *E. coli* TOP10 expressing OXA-48 from a pTOPO plasmid (6, 18) (Table 1).

A DNA fragment corresponding to the mature OXA-535, generated with the primers INF-OXA-48Fw (5'-AAGGAGATATACATATGGTAGCAAAGGAATGGCAAG-3') and INF-OXA-48Rv (5'-GGTGGTGGTCTCGAAGGGAATAATTTTCTCTGTTGAG-3'), was cloned into

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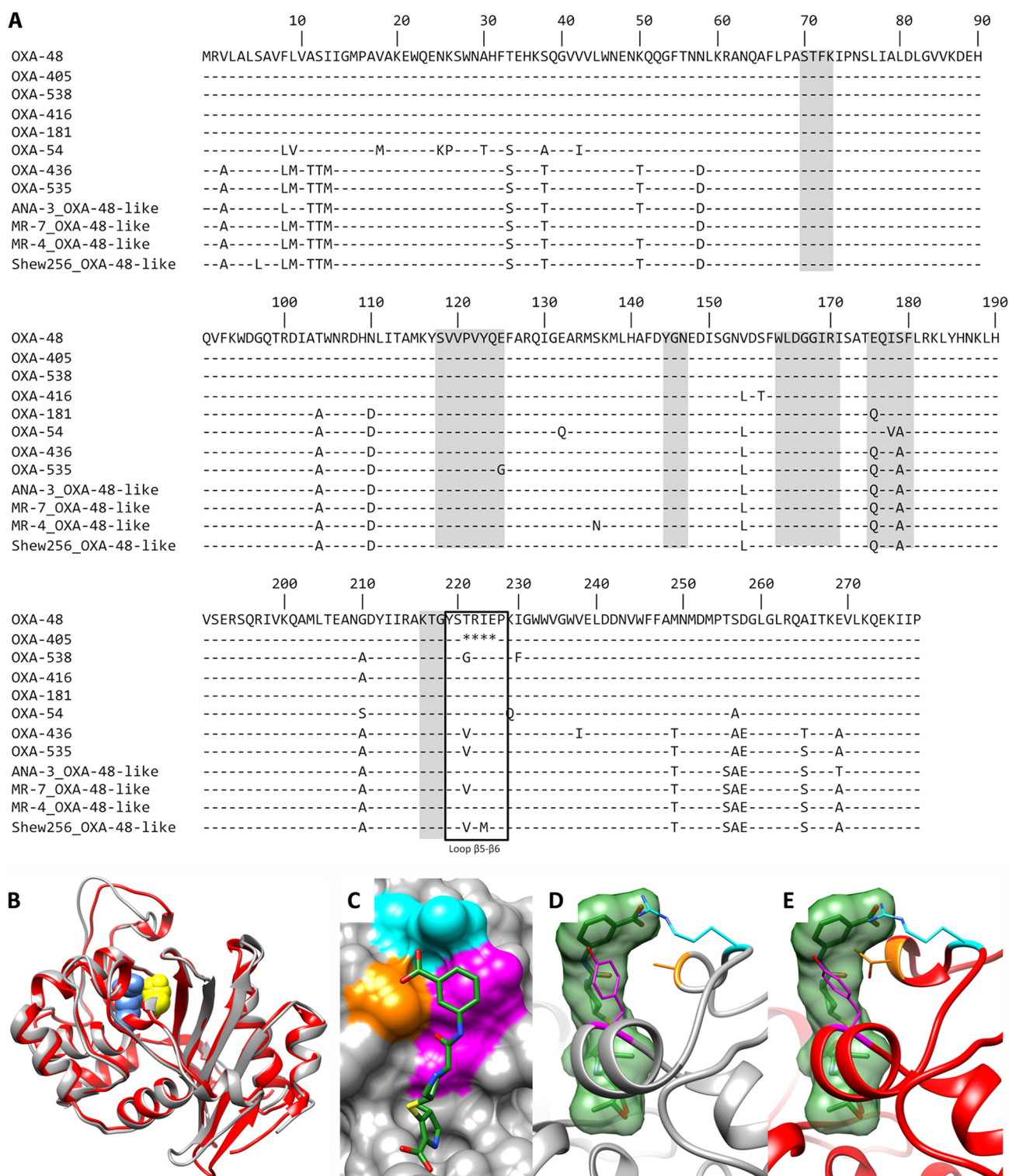


FIG 1 Amino acid sequence alignment of different OXA-48-like variants and molecular modeling. (A) Dashes indicate identical residues among all the amino acid sequences. Amino acid motifs that are well conserved among class D β -lactamases are indicated by gray boxes, and the single black-outlined box corresponds to the β 5- β 6 loop. Numbering is according to DBL (1). OXA sequences were from the β -lactamase database (<http://bldb.eu/BLDB.php?class=D#OXA>) (4) and ANA-3_OXA-48-like, MR-7_OXA-48-like, MR-4_OXA-48-like, and Shew256_OXA-48-like were from GenBank (accession nos. [WP_011718232.1](http://www.ncbi.nlm.nih.gov/nuccore/WP_011718232.1), [WP_011627184.1](http://www.ncbi.nlm.nih.gov/nuccore/WP_011627184.1), [WP_011621491.1](http://www.ncbi.nlm.nih.gov/nuccore/WP_011621491.1), and [WP_088210206.1](http://www.ncbi.nlm.nih.gov/nuccore/WP_088210206.1), respectively). (B) Superposition of crystal structures of OXA-535 (gray) and OXA-48 (red; PDB accession no. 4S2P). Residues Ser70 and Lys73 are shown in a Corey-Pauling-Koltun (CPK) representation and shown in yellow and blue, respectively. (C) Covalent docking pose of ertapenem (green, stick representation) in the binding site of OXA-535 (gray, surface representation). Residues Ala104, Arg107, and Tyr185 are orange, cyan, and magenta, respectively. Images were generated using Chimera (23). (D) Highlight of the favorable interaction between ertapenem (covalent docking pose) and residues Ala104, Arg107, and Tyr185 of OXA-535. (E) Highlight of the steric hindrance between ertapenem and the Thr104 residue of OXA-48 superimposed on the OXA-535 structure.

TABLE 1 MIC of β -lactams for *Shewanella* spp. JAB-1 and *E. coli* TOP10 pTOPO-OXA-535, TOP10 pTOPO-OXA-48, and TOP10

Antibiotic	MIC (mg/liter)			
	<i>Shewanella</i> sp.		<i>E. coli</i> TOP10	
	JAB-1	pTOPO-OXA-535	pTOPO-OXA-48	<i>E. coli</i> TOP10
Amoxicillin	>256	>256	>256	2
Amoxicillin + CLA ^a	8	128	>256	2
Piperacillin	>256	>256	>256	1.5
Temocillin ^b	0.25	>1,024	>1,024	4
Cefotaxime ^b	>32	0.06	0.75	0.06
Ceftazidime ^b	2	0.19	0.19	0.12
Cefepime	8	0.023	0.19	0.023
Imipenem ^b	0.38	1	0.75	0.25
Ertapenem ^b	1	0.25	0.25	0.003
Meropenem ^b	0.38	0.19	0.25	0.016

^aCLA, clavulanic acid (2 mg/liter).^bMIC data for these antibiotics were retested in this work but are similar to those presented by Jousset et al. (18).

the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois, France), and recombinant plasmid pET41-OXA-535 was electroporated into the electrocompetent *E. coli* strain BL21(DE3) (Novagen). OXA-535 was overexpressed and purified, and steady-state kinetic parameters were determined and compared with those of OXA-48 as previously described (6, 19).

Initial hydrolysis tests using pure undiluted OXA-535 revealed an absence of hydrolysis of penicillins (ampicillin, oxacillin, piperacillin, amoxicillin, and benzylpenicillin), whereas the other β -lactams tested were rapidly hydrolyzed by OXA-535 in a manner similar to that of OXA-48 (Table 2). The addition of 50 mM sodium hydrogen carbonate (NaHCO₃) as a source of CO₂ did not modify the rates of penicillin hydrolysis, suggesting that the absence of penicillinase activity with concentrated OXA-535 may not be due to Lys73 decarboxylation (20). When using 100-fold-diluted OXA-535 preparations, penicillin hydrolysis was restored in a manner similar to that of OXA-48. This peculiar behavior of OXA-535 in respect to penicillin hydrolysis may be the result of two mechanisms: (i) an alteration in the affinity of the enzyme for penicillins at high enzymatic concentrations and/or (ii) a modification in the hydrolysis mechanism that may involve an alternative catalytic process specific for penicillins, active only with low concentrations of the enzyme, which may be a result of enzyme concentration-dependent conformational changes. Structural analyses should be performed to explain this peculiar OXA-535 behavior.

Steady-state kinetic parameters for penicillins were determined by using diluted

TABLE 2 Comparison of the steady-state kinetic parameters of OXA-48 and OXA-535 β -lactamases

Substrate	K_m (μ M)		k_{cat} (s ⁻¹)		k_{cat}/K_m (mM ⁻¹ s ⁻¹)	
	OXA-48	OXA-535	OXA-48	OXA-535	OXA-48	OXA-535
Ampicillin	395	67	955	41	2,418	615
Oxacillin	95	53	130	64	1,368	1,210
Temocillin	45	357	0.30	1.8	6.6	5.1
Cefalotin	195	197	44	15	226	74
Cefoxitin	>200	268	>0.05	0.07	0.26	0.27
Ceftazidime	NH ^a	>1,000	NH	>0.01	NH	0.01
Cefotaxime	>900	>1,000	>9.0	>3.9	10	3.9
Cefepime	>550	>1,000	>0.60	>0.34	1.1	0.34
Imipenem	13	105	4.8	7.1	369	67
Meropenem	11	3.0	0.07	0.20	6.2	68
Ertapenem	100	0.06	0.13	0.17	1.3	2,646

^aNH, hydrolysis was not detected with concentrations of substrate and enzymes up to 1,000 and 400 nM, respectively.

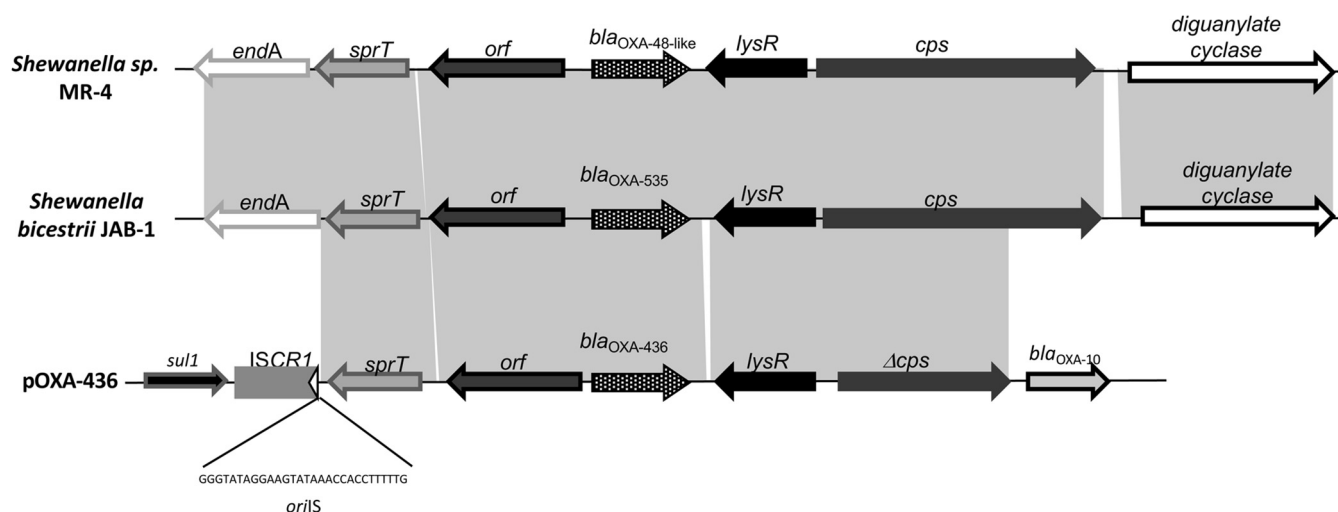


FIG 2 Genetic contexts of the *bla*_{OXA-535} gene. Major genetic features of the environment of the different *bla*_{OXA-48-like} genes in *Shewanella* spp., i.e., MR-4 (accession no. CP000446.1) and *S. bicestii* JAB-1 (accession no. NZ_CP022358.1), and of plasmid pOXA-436 (accession no. KY863418.1) isolated from *Enterobacter asburiae* strain AMA 497. Common features are highlighted in gray. *sprT* codes for a SprT-like protein of unknown function, *endA* encodes an endonuclease I, *lysR* encodes a putative *lysR*-type transcriptional regulator gene, and *cps* encodes a carbamoyl-phosphate synthase.

OXA-535 preparations. OXA-535 and OXA-48 presented similar catalytic efficiencies for oxacillin and temocillin (Table 2) (6). Compared with OXA-48, OXA-535 catalytic efficiency was (i) ~4-fold lower for ampicillin due to a lower turnover number; (ii) 3-fold lower for cephalothin due to a lower k_{cat} ; (iii) 5-fold lower for imipenem as a result of a lower affinity (8-fold increased k_m); (iv) 11-fold higher for meropenem as a result of better affinity and turnover values; and, most surprisingly, (v) ~1,800-fold higher for ertapenem, because of a 1,660-fold-higher affinity, with similar velocity of hydrolysis to that of OXA-48 (Table 2). This reduced activity against imipenem of OXA-535 may explain the negative results observed by Jousset et al. (18), who used Carba NP tests. The fact that OXA-535-like enzymes are difficult to detect with biochemical tests, such as Carba NP, may lead to the silent spread of this type of enzyme, in a manner similar to that of OXA-244 (18, 21).

Suitable conditions for the crystallization of OXA-535 were found at facilities of the Institute for Integrative Biology of the Cell (I2BC, Gif-sur-Yvette, France), and the resulting crystals were used for acquisition of X-ray diffraction data at the SOLEIL synchrotron (Saclay, France). A preliminary crystallographic study (data not shown) showed that the structures of OXA-535 and OXA-48 are very similar, with the exception of three regions situated relatively far from the binding site, i.e., 49 to 55, 94 to 106, and 252 to 261 (class D β -lactamase [DBL] numbering system) (Fig. 1B) (1). Covalent docking of ertapenem using GOLD and the GoldScore scoring function showed a strong ionic interaction between the terminal carboxylate group of the ligand and the side chain of Arg107 and a C-H... π interaction between the side chain of Tyr185 and the terminal aromatic group of ertapenem (Fig. 1C) (22). Comparison with the OXA-48 structure showed that the residue in position 104 plays a key role in ertapenem binding, thus explaining the huge difference in affinity between OXA-48 and OXA-535 determined for this substrate. Indeed, Ala104 in OXA-535 is compatible with ertapenem binding, given the small size of the side chain and the modified conformation of loop 94 to 106 (Fig. 1D), whereas Thr104 in OXA-48 is bulkier and completely blocks ertapenem binding in this region (Fig. 1E). These differences may have led to the observed 1,800-fold difference in catalytic efficiency.

Upstream of the *bla*_{OXA-535} gene, we identified *sprT* and *endA* genes encoding an SprT-like protein of unknown function and endonuclease I, respectively. The same genetic organization was described for closely related chromosomally encoded *bla*_{OXA-48-like} genes, such as the *bla*_{OXA-48-like-MR-4} gene (Fig. 2). The downstream

sequence of the *bla*_{OXA-535} gene is commonly present downstream of *bla*_{OXA-48-like} genes on the chromosomes of *Shewanella* spp. (11, 12). The 2,394-bp upstream and 4,074-bp downstream sequences of the *bla*_{OXA-535} gene were identical to those of *bla*_{OXA-436}, further supporting that the chromosomally encoded *bla*_{OXA-535} gene may be the progenitor of the plasmid-encoded *bla*_{OXA-436} gene (5). An *ISCR1* element found upstream of the *bla*_{OXA-436} gene but absent upstream of the *bla*_{OXA-535} gene may be responsible for the *bla*_{OXA-535-like} gene mobilization from the chromosome of *Shewanella* spp. to plasmids (Fig. 2). *ISCR1* carries a single *orf* that is responsible for a rolling-circle type of transposition mechanism (24). Flanking gene acquisition is thought to occur when the termination mechanism fails and rolling-circle replication extends into neighboring DNA, where it may encounter a second surrogate end (24). In the case of the *bla*_{OXA-436} gene, the DNA sequence putatively mobilized by *ISCR1*-mediated rolling-circle transposition corresponded to an ~6.5-kb fragment present also in *S. bicestii* JAB-1 and surrounding the *bla*_{OXA-535} gene. Our findings illustrate the increasing complexity of genetic vehicles at the origin of OXA-48-type carbapenemase spread (7–10). The finding of *ISCR1* upstream of *bla*_{OXA-48-like} genes is worrisome, because this *ISCR1* plays an important role in the assembly and transmission of multiple antibiotic resistance genes (5, 18, 24).

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We have no competing interests to declare.

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Unravelling ceftazidime-avibactam resistance of KPC-28, a KPC-2 variant lacking carbapenemase activity

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Abstract

Backgrounds: KPC-like carbapenemases have spread worldwide with more than thirty variants identified and differing by single or double amino-acid substitutions. Here, we describe the steady-state kinetic parameters of KPC-28 that differs from KPC-2 by a H274Y substitution and a two amino-acids deletion (Δ 242-GT-243).

Materials/Methods: The *bla*_{KPC-2}, *bla*_{KPC-3}, *bla*_{KPC-14} and *bla*_{KPC-28} genes were cloned into pTOPO vector for susceptibility testing or into pET41b for over-expression, purification and subsequent kinetic parameters (K_m , k_{cat}) determination. Molecular docking experiments were performed to explore the role of the amino-acid changes in the carbapenemase activity.

Results: Susceptibility testing revealed that *E. coli* producing KPC-28 displayed MICs that were lower for carbapenems and higher for ceftazidime and ceftazidime/avibactam as compared to KPC-2. The catalytic efficiencies of KPC-28 and KPC-14 for imipenem were 700-fold and 200-fold lower, respectively, than those of KPC-2, suggesting that the Δ 242-GT-243 in KPC-28 and KPC-14 is responsible for reduced carbapenem hydrolysis. Similarly, the H274Y substitution resulted in a 50-fold increase in ceftazidime hydrolysis that was strongly reversed by clavulanate.

Conclusion: Here, we have shown that KPC-28 is lacking carbapenemase activity, has increased ceftazidime hydrolytic activity, and is strongly inhibited by clavulanate. KPC-28-producing *E. coli* isolates display an avibactam-resistant ESBL profile, that may be wrongly identified by molecular and immunochromatographic assays as a carbapenemase. Accordingly, confirmation of carbapenem hydrolysis will be mandatory with assays based solely on *bla*_{KPC} gene or gene product detection.

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INTRODUCTION

In Gram negative bacteria, acquired resistance to β -lactams may be mediated by various non-enzymatic mechanisms such as decreased permeability of the outer-membrane, or active efflux, but enzymatic inactivation by β -lactamases is the main mechanism. These enzymes, which are able to hydrolyse β -lactams, are divided into 4 molecular classes based on structural homologies (Ambler classification). Ambler's classes A, C and D are β -lactamases possessing a serine in their active site whereas the class B enzymes are metallo- β -lactamases (MBLs) that use divalent Zn^{2+} ion(s) for their hydrolytic activity. During the last decade, the rising use of carbapenems (imipenem and meropenem) for the treatment of infections caused by multidrug resistant Gram negative pathogens led to the emergence of carbapenem-hydrolysing β -lactamases, called carbapenemases.¹ These enzymes belong to Ambler's classes A, B and D.² Within class A, several enzymes were described in Enterobacteriaceae (NMC-A, IMI-1, SME-1, GES-2, FRI-1) but *Klebsiella pneumoniae* carbapenemase (KPC) is the most prevalent in the world. KPC is considered as the most worrisome class A carbapenemase because of its location on self-conjugative plasmids, and its frequent association with a highly successful *K. pneumoniae* clone, the clonal complex (CC) 258.³ Until 2005, the geographic distribution of KPC-producing Enterobacteriaceae remained limited to the east of United States.⁴ Nowadays, KPC-producers have disseminated worldwide and are endemic in the United States, South America, Greece, Italy, Poland, China and Israel. Since its initial description in a *K. pneumoniae* clinical isolate from North Carolina,⁵ thirty-seven KPC-variants have been reported.⁶ Among these variants, a mono-substitution (KPC 3-6 and 9-11) resulted in increased ceftazidime hydrolysis, with carbapenem hydrolysis being unaffected.⁷⁻¹¹ Recently, *in vitro* selection of ceftazidime-avibactam (CAZ-AVI) resistance in Enterobacteriaceae with KPC-3 carbapenemase revealed modifications in the Ω loop that are also responsible for carbapenem susceptibility. The most prevalent modification occurs at position 179 of the KPC enzyme,^{12,13} but few other substitutions at different positions were also involved such as V240G and T243A.¹² CAZ-AVI resistance also occurred *in vivo* usually following CAZ-AVI treatment for prolonged periods (> 2 weeks).¹⁴⁻¹⁷ The D179 modification is always reported in case of *in vivo* selection of CAZ-AVI resistant KPC-producing isolates.^{14,16} Recently, KPC-28, a new variant of KPC-2 with a two amino-acid deletion (Δ 242-GT-243, according to the ABL numbering scheme)¹⁸ and a substitution H274Y, was reported.¹⁹ In this study, a KPC-28-producing clone was found to be more resistant to ceftazidime as compared to KPC-2- or KPC-3-producers, but fully susceptible to carbapenems, suggesting a complete loss of carbapenemase activity. Here, we report the susceptibility profile and the steady-state kinetic characterization of the KPC-28 β -lactamase in comparison with KPC-2, KPC-3 (H274Y substitution) and KPC-14 (Δ 242-GT-243).

MATERIAL AND METHODS

Bacterial strains.

The clinical strain *Escherichia coli* W12 expressing the KPC-28 β -lactamase was used for cloning of the *bla*_{KPC-28} gene.¹⁹ *E. coli* TOP10 (Invitrogen, Saint-Aubin, France) was used for cloning and mutagenesis experiments, and *E. coli* BL21 Rosetta-gamiTM DE3 (Novagen-Merck, Fontenay-sous-Bois, France) was used for overexpression experiments.

Susceptibility testing.

Antimicrobial susceptibilities were determined by the disk diffusion technique on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the EUCAST breakpoints, updated in May 2018 (<http://www.eucast.org>). MICs were determined using the Etest technique (bioMérieux, La Balme les Grottes, France).

PCR, cloning experiments, site-directed mutagenesis and DNA sequencing.

Whole-cell DNAs of *E. coli* isolates producing KPC-2, KPC-3, and KPC-28 were extracted using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France) and were then used as a template to amplify the *bla*_{KPC-2-like} genes. The gene encoded for KPC-14 was obtained by site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies), using the primer KPC-Y274H (5'-CTAACAAGGATGACAAGCCACAGCGAGGCCGTCATC-3') and the plasmid pTOPO-*bla*_{KPC-28} as a template. The PCR, using the primers Kpc-rbs (5'-CTCCACCTTCAAACAAGGAAT-3') and Kpc-rev (5'-ATCTGCAGAATTCGCCCTTCGCCATCGTCAGTGCTCTAC-3'), was able to amplify *bla*_{KPC-3} and *bla*_{KPC-28} genes. The amplicons obtained were then cloned into the pCR-Blunt II-Topo plasmid (Invitrogen) downstream from the pLac promoter, in the same orientation for the phenotypic studies. The recombinant pTOPO-KPC- plasmids were electroporated into the *E. coli* TOP10 strain. For protein production, the sequences without the peptide signal of the *bla*_{KPC-2}, *bla*_{KPC-3}, *bla*_{KPC-14} and *bla*_{KPC-28} genes were obtained by PCR amplification using primers NdeI-KPC-2₃₀₋₂₉₃ (5'-CATATGGCGGAACCATTCGCTAAC-3') and KPC-2 Δ STOP (5'-CTCGAGCTGCCCCGTTGACGCCAAT-3') and were then inserted into plasmid pET41b (Novagen). The recombinant plasmids were transformed into *E. coli* BL21 Rosetta-gamiTM DE3 (Novagen). All the recombinant plasmids were sequenced using a T7 promoter and M13 reverse primers or T7 terminator depending of the plasmid with an automated sequencer (ABI Prism 3100; Applied Biosystems). The nucleotide sequences were analysed using software available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

Detection of KPC producers and carbapenemase activity.

The detection of the KPC-variants was performed with *E. coli* TOP10 harbouring the recombinant vector pTOPO-KPC. The detection of the carbapenemase activity was done by using four techniques: The Carba NP test as previously described²⁰, the β -CARBATM test (BioRad)²¹, the RAPIDEC® CARBA NP (bioMérieux)²² and the MBT STAR®-Carba IVD Kit (Bruker Daltonics, Bremen, Germany)²³ according to the manufacturer's recommendations. Molecular tests were performed with the kit Xpert® Carba-R (Cepheid, Sunnyvale, USA)²⁴ and by standard in-house PCR using the primers KPC-A (5'-CTGTCTTGTCTCTCATGGCC-3') and KPC-B (5'-CCTCGCTGTGCTTGT-CATCC-3'). Immunoenzymatic tests were carried out with two techniques: NG- CARBA 5 (NG Biotech, Guipry, France)²⁵ and the K-Set Resist-4 O.K.N.V. (CORIS BioConcept, Gembloux, Belgium).²⁶

Protein purification

An overnight culture of *E. coli* BL21 Rosetta-gamiTM DE3 harbouring recombinant pET41b-KPC- plasmids was used to inoculate 2 L of LB medium broth containing 50 mg/L kanamycin

and 30 mg/L chloramphenicol. Bacteria were cultured at 37°C until an OD of 0.6 at 600 nm was reached. The expression of the β -lactamase genes was carried out overnight at 22°C with 1 mM IPTG as inducer. Cultures were centrifuged at 6000 g for 15 min and then the pellets were resuspended with the binding buffer (10 mM Imidazole, 25mM Sodium phosphate pH 7.4 and 300 mM NaCl). Bacterial cells were disrupted by sonication and the bacterial pellet was removed by two consecutive centrifugation steps at 10,000 g for 1h at 4°C and then the supernatant was centrifuged at 96 000 g for 1h at 4°C. The soluble fractions were filtered and then passed through a HisTrapTM HP column (GE Healthcare), proteins were eluted with the elution buffer (500 mM Imidazole, 25mM Sodium phosphate pH 7.4 and 300 mM NaCl). Finally, a gel filtration step was performed with 100 mM sodium phosphate buffer pH 7 and 150 mM NaCl with a Superdex 75 column (GE Healthcare). The protein purity was estimated by SDS-PAGE and the pooled fractions were dialyzed against 10 mM Tris-HCl pH 7.6 and concentrated using Vivaspin columns. The concentrations were determined by measuring the optical density at 280 nm and with the extinction coefficients obtained from the ProtParam tool (Swiss Institute of Bioinformatics online resource portal).²⁷

Steady state kinetic parameters

Kinetic parameters were determined using purified KPC-2, KPC-3, KPC-14 and KPC-28 β -lactamases in 100 mM sodium phosphate buffer (pH 7). The k_{cat} and K_m values were determined by analysing β -lactam hydrolysis under initial-rate conditions with an ULTROSPEC 2000 UV spectrophotometer and the SWIFT II software (GE Healthcare, Velizy-Villacoublay, France) using the Eadie-Hoffstee linearization of the Michaelis-Menten equation. The different β -lactams were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). For some cephalosporins, saturation could not be reached. Thus, the values for the catalytic efficiency (k_{cat}/K_m) of the enzymes KPC-2 and KPC-3 against these substrates were evaluated with the lower limits for the k_{cat} and K_m determined. Fifty percent inhibitory concentration (IC50) for the β -lactamase inhibitor clavulanic acid was determined in 100 mM sodium phosphate buffer (pH 7) and with 100 μ M of piperacillin as a reporter substrate.

Molecular Modelling

Molecular models of KPC-3, KPC-14 and KPC-28 were generated by comparative modelling using MODELLER version 9.16²⁸ with the KPC-2 structure²⁹ (PDB code 5UJ3) as template. Three-dimensional structures of the ligands were generated using CORINA version 3.60 (Molecular Networks GmbH, Erlangen, Germany, <http://www.molecular-networks.com>). Covalent docking calculations were carried out using GOLD version 5.2³⁰ and the GoldScore scoring function. The binding site was defined as a sphere with a 20 Å radius around the OG atom of the Ser70 residue. The covalent connection was made between the OG atom of the Ser70 residue and the open form of the β -lactam ring in order to generate the acyl-enzyme complex. Molecular modelling images were generated using UCSF CHIMERA.³¹

RESULTS

Clinical isolate

E. coli W12 was recovered from a fecal sample from a Portuguese patient upon admission at a French hospital subsequent to a medical repatriation. This isolate was resistant to broad-spectrum cephalosporins, carbapenems, aminoglycosides and colistin.¹⁹ As previously described, this isolate possessed the *bla*_{OXA-48} and *bla*_{KPC-28} genes and the acquired colistin resistance determinant *mcr-1*.

¹⁹ KPC-28 differed from KPC-2 by a substitution (H274Y) and a deletion of 2 amino acids (Δ 242-GT-243) (Figure 1).

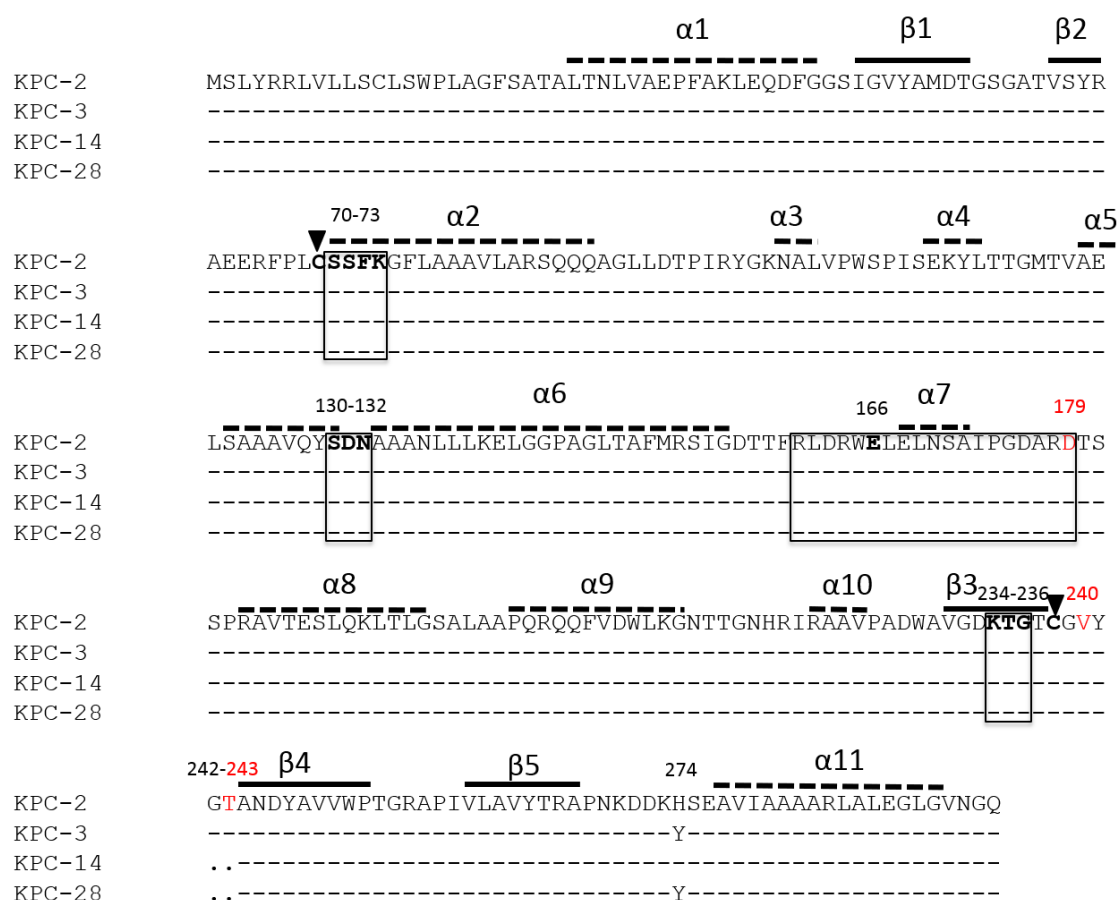


Figure 1. Sequence alignments of KPC variants. Alpha helices are indicated by dashed lines and beta strand by continuous black lines. Cysteines involved in disulfide bond are indicated by black triangles. Key residues known to be implicated in ceftazidime/avibactam resistance are indicated in red. Conserved residues among class A β -lactamases are indicated by boxes.

Antimicrobial susceptibilities of transformants with KPC-2, KPC-3, KPC-14 and KPC-28.

To evaluate and compare the antimicrobial susceptibility profiles conferred by KPC-28, the *bla*_{KPC-2}, *bla*_{KPC-3}, *bla*_{KPC-14} and *bla*_{KPC-28} genes were cloned into pTOPO vector and expressed in *E. coli* TOP10 (Table 1). Although the different KPC variants exhibited similar MICs for amoxicillin, temocillin, cefixime and cefepime, KPC-28- and KPC-14-producing *E. coli* TOP10 possessed lower MICs for cefotaxime and carbapenems (being fully susceptible) but exhibited higher MIC values for ceftazidime as compared to KPC-2- and KPC-3-producers (Table 1). Of note, the highest MICs for cefotaxime and aztreonam were found with the KPC-3-producing *E. coli* TOP10 clones. The addition of clavulanic acid restored susceptibility to amoxicillin for KPC-28- and KPC-14-producing *E. coli* clones, while inhibition by

Table 1. MIC profile of *E. coli* TOP10 expressing KPC-2, KPC-3, KPC-14 and KPC-28 β -lactamases

Antimicrobial agent	MIC (mg/L)				
	<i>E. coli</i> TOP10 pTOPO KPC-2	<i>E. coli</i> TOP10 pTOPO KPC-3	<i>E. coli</i> TOP10 pTOPO KPC-14	<i>E. coli</i> TOP10 pTOPO KPC-28	<i>E. coli</i> TOP10
Amoxicillin	> 256	> 256	> 256	> 256	6
Amoxicillin + CLA ^a	24	48	6	6	6
Temocillin	12	16	16	16	6
Ceftriaxone	12	48	6	4	0.032
Cefotaxime	8	> 32	6	4	0.064
Ceftazidime	4	> 256	> 256	> 256	0.125
Ceftazidime + Avibactam ^b	0.38	0.75	24	12	0.125
Cefixime	3	12	12	8	0.38
Cefepime	2	6	4	4	0.0064
Aztreonam	24	> 256	32	24	0.047
Imipenem	8	8	0.25	0.25	0.25
Meropenem	3	3	0.032	0.032	0.032
Ertapenem	1	1	0.006	0.008	0.004

^aCLA, clavulanic acid at a fixed concentration of 4 mg/L, ^b Avibactam at a fixed concentration of 4 mg/L

avibactam seemed to be less efficient, as both KPC-28- and KPC-14-producing *E. coli* isolates were resistant to the association ceftazidime/avibactam. Thus, MIC values suggested that the Δ 242-GT-243 (in KPC-14 and KPC-28) resulted in the loss of carbapenemase activity but in increased hydrolytic activity towards ceftazidime. This increased ceftazidime hydrolytic activity was potentiated by the H274Y substitution in KPC-28, as already shown for KPC-3, but in the latter case, carbapenem-resistance was not affected.¹¹

Biochemical properties of KPC-28

To characterize and compare kinetic parameters of KPC-28, the four variants have been purified and kinetic studies were performed (Table 2). KPC-2 was used as control for comparison. Overall, the steady-state kinetics revealed three patterns. KPC-3 exhibited a higher hydrolytic activity towards expanded-spectrum cephalosporins (cefotaxime, ceftazidime and cefepime) but a similar hydrolysis rate for carbapenems as compared to KPC-2. In comparison, KPC-14 and KPC-28 possessed a higher affinity for ceftazidime resulting in increased catalytic efficiencies of 40-fold and 50-fold, respectively, with however reduced carbapenemase activity, as revealed by the 200-fold and 700-fold reduction in imipenem catalytic efficiencies, respectively, as compared to KPC-2. Thus, the reduced carbapenem hydrolysis can be linked to the Δ 242-GT-243 that results in a 10-fold increase in affinity but also a

Table 2. Steady state kinetic parameters for hydrolysis of β -lactam substrates by KPC-2 and KPC-2-like β -lactamases

Substrate	K_m (μ M)				k_{cat}				k_{cat}/K_m			
	KPC-2	KPC-3	KPC-14	KPC-28	KPC-2	KPC-3	KPC-14	KPC-28	KPC-2	KPC-3	KPC-14	KPC-28
Piperacillin	137	255	6	11	59	38	0.8	0.95	434	150	127	86
Cefoxitine	>1000	>1000	NH	NH	>12	>4	ND	ND	2	1.6	ND	ND
Cefotaxime	>1000	335	77	75	>163	403	4	5	95	1202	52	67
Ceftazidime	>1000	656	41	125	>16	9	1	4	0.6	14	24	32
Cefepime	>1000	491	34	30	>48	14	1.7	2.5	13	29	50	83
Imipenem	198	235	5	18	47	31	0.006	0.006	237	131	1.2	0.34
Meropenem	45	18	7	NH	3	2	0.003	ND	67	103	0.4	ND
Ertapenem	30	25	5	9	4	3	0.002	0.004	133	114	0.47	0.42

ND, not determined; NH, no detectable hydrolysis.

Data are the mean of three independent experiments.^[1] Standard deviations were within 10% of the mean value.^[2]

1000-fold decrease in k_{cat} values. Similarly, KPC-14 and KPC-28 lost completely cefoxitin hydrolytic activity. IC₅₀ values of clavulanic acid for KPC-2, KPC-3, KPC-14 and KPC-28 were 47 mg/L, 16 mg/L, 0.1 mg/L and 0.09 mg/L, respectively. Taken together, our results suggest that KPC-28 displays a clavulanic acid inhibited ESBL profile and not that of a carbapenemase anymore. Finally, our data suggest that the increased ceftazidime hydrolysis of KPC-28 is due to the H274Y substitution as shown in this study (Table 2) confirming previous results,¹¹ but is potentiated by the 2 amino-acid deletion (Δ 242-GT-243). The H274Y substitution does not affect carbapenem-hydrolysis, unlike the Δ 242-GT-243 deletion (Table 2).

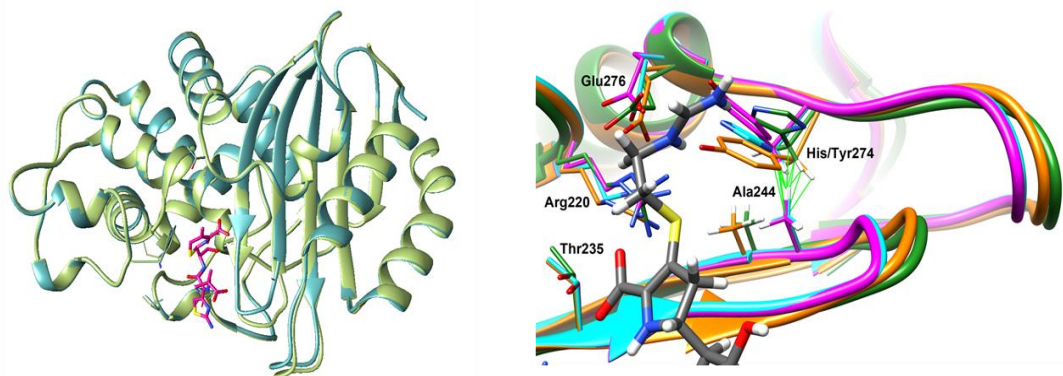
Molecular modelling

We performed a molecular modelling study to identify the structural determinants that could explain the experimentally-determined differences between the hydrolytic profiles of KPC-2, KPC-3, KPC-14 and KPC-28. In the absence of structural data for the KPC-2 variants, we have generated molecular models of KPC-3, KPC-14 and KPC-28 by comparative modelling using MODELLER version 9.16²⁸ with the KPC-2 structure (PDB code 5UJ3) as template (Figure 2A, 2B).²⁹ The resulting models showed that the Δ 242-GT-243 deletion didn't modify the overall structure of the protein, but resulted in a shorter 238-243 loop, giving rise to a 2.4 Å shift of A244 in KPC-14 and KPC-28 (Figure 2A). This new position of A244 led to a clash between the side chains of A244 and of the residue at position 274 (H and T for KPC-14 and KPC-28, respectively) that may expand the active site and allow a better access for the substrates. In addition, the covalent complex of KPC-2 with imipenem obtained by docking showed a stabilizing interaction between H274 and the positively charged R2 substituent of imipenem (Figure 2B). In the case of KPC-14 and KPC-28, the above-mentioned clash may prevent this stabilizing interaction with imipenem. In these conditions, the substrate may interact with the binding site in a slightly different mode and therefore explain the 10-fold increase in K_m and the 1000-fold decrease in k_{cat} , which ultimately lead to the loss of the carbapenemase activity in these mutants. Additional theoretical calculations, and especially molecular dynamics simulations, will be needed to understand these details.

Detection methods for KPC variants

Four carbapenemase detection tests based on imipenem hydrolysis were evaluated in respect to their ability to detect these four variants when expressed in *E. coli* TOP10 (Table 3). Thus, the Carba NP Test, the RAPIDEC[®] CARBA NP, the MBT STAR[®]-Carba IVD and the β -CARBA[™] test were able to detect KPC-2 and KPC-3,

2. A.



2. C.

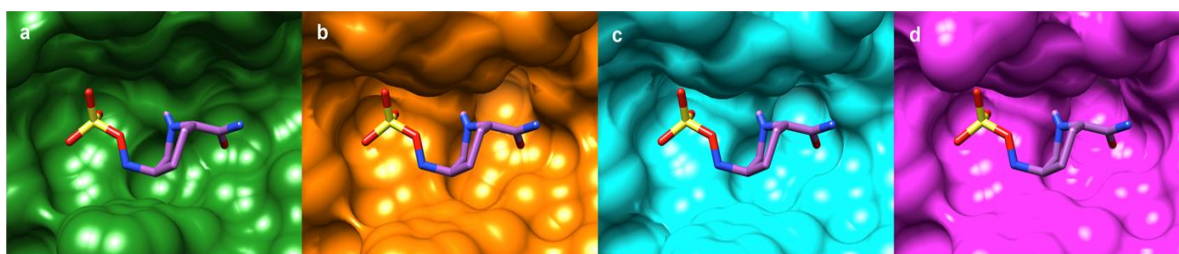


Figure 2. Models of interaction of KPC-3 variant (KPC-14 and KPC-28) with ceftazidime and imipenem. **A.** Superposition of the molecular model of KPC-3 and KPC-28 with ceftazidime. KPC-3 is in light green, KPC-28 in dark green and ceftazidime in magenta. **B.** Crystal structure of KPC-2 (PDB code 5UJ3, green) superposed with homology models of KPC-3 (orange), KPC-14 (cyan) and KPC-28 (magenta). The imipenem conformation obtained by covalent docking on KPC-2 is shown as grey sticks. The steric clashes between the side chains of A244 and of the residue at position 274 are highlighted in light green. **C.** Crystal structure of KPC-2 in complex with avibactam (**a**, PDB code 4ZBE, green) superposed with the KPC-3 (**b**, orange), KPC-14 (**c**, cyan) and KPC-28 (**d**, magenta) homology models, showing no significant clashes between the protein and the ligand.

but failed to detect KPC-14 and KPC-28.²⁰⁻²³ These results are in line with the kinetic studies, which showed a loss of carbapenemase activity for KPC-14 and KPC-28. On the other hand, immunochromatographic assays RESIST-4 O.K.N.V kit (CORIS BioConcept, Gembloux, Belgium) and the NG-Test CARBA 5 (NG Biotech, Guipry, France) and molecular Xpert® Carba-R test (Cepheid, Sunnyvale, USA) and in-house PCR were positive for all four enzymes.^{24-26,32} The positive results of the immunochromatographic assay for KPC-14 and KPC-28 confirmed our molecular modelling results, suggesting that the $\Delta 242\text{-GT-}243$ did not affect the global conformation of these proteins.

Table 3. Diagnostic tests performed on *E. coli* TOP10 harbouring the vector pTOPO expressing KPC-2, KPC-3, KPC-14 and KPC-28.

	Biochemical tests				Molecular tests		Immunoenzymatic tests	
	Carba NP test	RAPIDEC® CARBA NP	β CARBA™	MBT STAR-Carba IVD Kit	Standard <i>bla</i> _{KPC} PCR	Xpert Carba-R	NG-Test CARBA 5	Coris Resist-4 O.K.N.V. K-Set
KPC-2	+	+	+	+	+	+	+	+
KPC-3	+	+	+	+	+	+	+	+
KPC-14	-	-	-	-	+	+	+	+
KPC-28	-	-	-	-	+	+	+	+

+, positive test; -, negative test

DISCUSSION

KPC-producing Enterobacteriaceae are now endemic in the United State and have spread worldwide. So far, thirty-five variants of KPC have been reported⁶, with KPC-2 and KPC-3 appearing to be the most prevalent.³³ In this study, we have characterized the biochemical properties of KPC-28, which shared with KPC-3 the same substitution H274Y and possesses in addition a Δ242-GT-243 deletion previously identified in KPC-14. Steady-state kinetics of KPC-2, KPC-3, KPC-14 and KPC-28 β-lactamases revealed that the unique substitution of KPC-3 (H274Y) conferred a 20-fold increase of the catalytic efficiency towards ceftazidime as compared with KPC-2. This substitution was also identified in other variants such as KPC-7 (M49I; H274Y), KPC-8 (V240G; H274Y), KPC-9 (V240A; H274Y) and KPC-10 (P104R; H274Y), which are associated with increased ceftazidime resistance. The study of KPC-5 showed that substitution of the residues 104 can also confer an increased ceftazidime hydrolysis.¹⁰ These studies highlighted that a single or a double substitution can affect the hydrolysis rate of cephalosporins but do not change significantly the carbapenemase activity. More recently, Shields *et al.* reported that T243A substitution increased ceftazidime hydrolysis of the KPC-3 enzyme with little impact on carbapenemase activity.¹² Here, we demonstrated that the Δ242-GT-243 deletion also led to an increase of the ceftazidime hydrolysis, but unlike T243A substitution, has a drastic impact on carbapenemase activity. For example, compared to KPC-2, the catalytic efficiencies (k_{cat}/K_m) towards imipenem were 700-fold and 200-fold lower for KPC-28 and KPC-14, respectively. This deletion is linked with a 10-fold higher affinity (lower K_m) but a 1000-fold decreased k_{cat} for carbapenems. The loss of the carbapenemase activity can be explained by the loss of the interaction between the residue H274 in KPC-14 and Y274 in KPC-28 and carbapenems due to a 2Å shift of the A244. Additionally, the 50% inhibitory concentration (IC50) of clavulanate was 500-fold lower with KPC-28 as compared to KPC-2. The most worrisome result is that this 2 AA deletion has also an impact on ceftazidime/avibactam susceptibility. Indeed, while KPC-2 and KPC-3-producing *E. coli* isolates remain susceptible to ceftazidime/avibactam, KPC-14 and even more so

KPC-28-producing *E. coli* isolates are resistant. Several mutations have been identified to yield ceftazidime/avibactam resistance with KPC enzymes. The most common *in vivo* mutation described is D179Y especially associated with the H279Y mutation that yields in increased ceftazidime hydrolysis.³⁴ Other mutations, with minor phenotypic expressions have also been identified, such as S130G, T243A or T243M.³⁴ In KPC-28, T243 has been deleted and may thus play also an additional role in avibactam resistance. It is very likely that avibactam resistance in KPC-28 is the result of the increased hydrolytic activities against ceftazidime since no clashes were evidenced between avibactam and the homology models of the KPC-3, KPC-14 and KPC-28 variants (Figure 2C).³⁴

CONCLUSION

This study underlines that KPC-type β -lactamases are more complex and diverse than expected. As exemplified by KPC-28 and KPC-14, they are not all true carbapenemases, a scenario well known for OXA-48-like enzymes.³⁵ Unfortunately, molecular detection assays and immunochromatographic tests are not able to discriminate KPC variants with carbapenem hydrolytic capacities from those lacking any carbapenemase activity. Therefore, the first-line screening of carbapenemase producers in *Enterobacteriaceae* have to include a test able to detect a carbapenemase activity, such as biochemical tests (e.g. Carba NP test and derivatives, β -CARBA™ test), or MALDI-TOF-based assays (e.g. STAR®-Carba IVD kit, Bruker). Finally, as KPC producing organisms cause infection with a high morbidity and mortality, avibactam was designed as a powerful inhibitor of KPC enzymes.³⁶⁻³⁹ However, several studies report now resistance to ceftazidime-avibactam of KPC-producing isolates as a consequence of selection of point mutant derivatives.³⁹ KPC-28-producing bacterial isolates, are resistant to ceftazidime/avibactam as a consequence of increased ceftazidime hydrolysis, rather than intrinsic avibactam resistance. Finally, as KPC-28 is lacking carbapenemase activity, has increased ceftazidime hydrolytic activity, and is strongly inhibited by clavulanate, KPC-28-producing bacterial isolates may be identified as ESBL producers.

FUNDING

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TRANSPARENCY DECLARATIONS

LD is co-inventor of the Carba NP Test, which patent has been licenced to bioMérieux (La Balme les Grottes, France).

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Section 2: Organizing and exploiting the available information on β -lactamases

Antimicrobial resistance poses a threat to public health, which must be faced given that the use of antimicrobials is a cornerstone of modern medicine as we know it. The most used antibiotics are β -lactams, and the most common mechanism of resistance to them is the expression of β -lactamases. The amount of information generated by the genetic, biochemical and structural study of β -lactamases since their discovery can be exploited for better understanding of the structure-function relationship and its implications for guiding the rational design of novel drugs that inhibit these enzymes or escape hydrolysis by them. However, the amount of data available is huge, and its dispersion over different databases and journals can make it difficult to recompile and profit from it. We combined all these data to develop a comprehensive and well-organized database “Beta-lactamase Data Base”, which should allow us to carry out a deeper and more efficient analysis of it.

ORIGINAL ARTICLE



Beta-lactamase database (BLDB) – structure and function

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ABSTRACT

Beta-Lactamase Database (BLDB) is a comprehensive, manually curated public resource providing up-to-date structural and functional information focused on this superfamily of enzymes with a great impact on antibiotic resistance. All the enzymes reported and characterised in the literature are presented according to the class (A, B, C and D), family and subfamily to which they belong. All three-dimensional structures of β -lactamases present in the Protein Data Bank are also shown. The characterisation of representative mutants and hydrolytic profiles (kinetics) completes the picture and altogether these four elements constitute the essential foundation for a better understanding of the structure-function relationship within this enzymes family. BLDB can be queried using different protein- and nucleotide-based BLAST searches, which represents a key feature of particular importance in the context of the surveillance of the evolution of the antibiotic resistance. BLDB is available online at <http://blldb.eu> without any registration and supports all modern browsers.

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antibiotic resistance;
hydrolytic profile; mutant

Introduction

β -Lactams, due to their safety, reliable killing properties and clinical efficacy, are among the most frequently prescribed antibiotics used to treat bacterial infections. However, their utility is being threatened by the worldwide proliferation of β -lactamases (BLs) with broad hydrolytic capabilities, especially in multi-drug-resistant gram-negative bacteria. These BLs are divided into four classes based on their sequence identities¹. While a handful of BLs were known in the early 1970s, their number has ever since been growing rapidly, especially with the description in clinical isolates of novel enzymes being capable of hydrolysing carbapenems, last resort antibiotics². A representative example is the class A KPC-2 that in a few years became one of the most menacing BL currently spreading worldwide³.

Historically, the principal resource of BLs was maintained from 2001 at the Lahey Clinic (<http://www.lahey.org/Studies/>) by George Jacoby and Karen Bush, by assigning new enzyme numbers for a number of representative BL families. From July 2015, this resource was transferred into the Bacterial Antimicrobial Resistance Reference Gene Database (<https://www.ncbi.nlm.nih.gov/bioproject/313047/>) maintained at the NCBI. Other resources are the Institute Pasteur MLST Database (<http://bigsd.b.pasteur.fr/klebsiella/klebsiella.html>), the Antibiotic Resistance Genes Database⁴, the Lactamase Engineering Database^{5,6}, the Metallo- β -Lactamase Engineering Database⁷, the Comprehensive Antibiotic Resistance Database⁸, the β -Lactamase Database⁹, the Comprehensive β -Lactamase Molecular Annotation Resource¹⁰. However, most of these databases are either not maintained

anymore, have a very broad scope or are focused on a few BL families.

The aim of our *Beta-Lactamase Database (BLDB)* is to compile sequence information as well as biochemical and structural data on all the currently known BLs. This comprehensive web-based database, which is updated on a weekly basis, may provide at a glance useful insights in the structure-function relationships of BLs, allowing a better understanding of substrate specificities and key residues involved in substrate recognition and hydrolysis. Altogether, the information provided by BLDB may help to foresee the impact of future mutations on the evolution of BLs.

Implementation details

The database is hosted on a dedicated virtual server in the cloud, which allows easy adjustments and evolution of computing resources according to the needs.

The core pages are implemented in PHP on a Linux Server under the CentOS 7.2 operating system, whereas the raw data is stored as tabulated files in order to facilitate the updates.

The interactive images showing the list of BL families that are present in the BLDB are generated dynamically in SVG format from the raw data, thus ensuring an updated display at any time. The corresponding URL links are directly embedded in the SVG images.

Multiple sequence alignments are automatically generated with Clustal Omega¹¹ using the default parameters. Phylogenetic trees are processed using Phylip version 3.695

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 Supplemental data for this article can be accessed [here](#).

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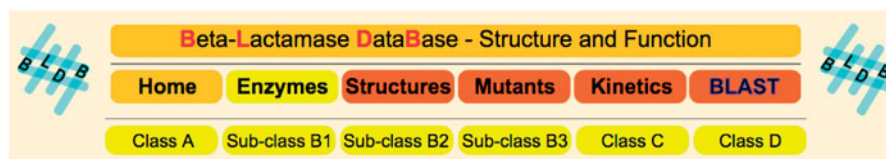


Figure 1. Global architecture of the Beta-Lactamase Database. In addition to the *Home* page, there are five main sections, dedicated to *Enzymes* (classified into the four classes A, B, C and D, and further into the three sub-classes of class B), three-dimensional *Structures* available in the Protein Data Bank, synthetic *Mutants* and hydrolytic profiles (*Kinetics*) described in the literature, and a graphical interface for *BLAST* queries.

(<http://evolution.genetics.washington.edu/phylip/>) using Clustal Omega's *DND* output files and represented as *SVG* images to provide the best quality and minimal file size.

The radar charts representing hydrolytic profiles are dynamically built using a modified and personalised version of the *D3.js* JavaScript library (<https://gist.github.com/nbremer/6506614>).

The BLAST interface is provided by SequenceServer¹² and the BLAST+ binaries are downloaded from NCBI¹³.

Input data is downloaded from NCBI using the "Entrez Direct: E-utilities on the UNIX Command Line"¹³ and from the PDB with personalised scripts.

Structures are updated semi-automatically on a weekly basis, after each PDB update. New enzymes are added following every update of the Bacterial Antimicrobial Resistance Reference Gene Database. Constant literature survey also provides newly described BLs and synthetic mutants, as well as their hydrolytic profiles. The long-term maintenance of the BLDB is ensured by the collaboration between two academic teams with active interest and experience in the field of BL-mediated antibiotic resistance.

Database architecture

BLDB is designed around five main sections, which are strongly interconnected and gathered around the main home page (Figure 1). All pages contain (i) a header showing the overall structure of the BLDB, with links for an easy access to all sections at any moment, and (ii) a footer with acknowledgments to funding bodies that have contributed to this project and with contact details.

Home page

A short introduction to the present challenges associated with the antibiotic resistance is presented, highlighting the important contribution provided by the BLDB in this field.

Real-time statistics with the number of entries for each type of data present in the BLDB (*Enzymes*, *Structures*, *Mutants* and *Kinetics*) and for each one of the four classes of BL are also provided. The entries corresponding to the subclasses B1, B2 and B3 of class B are further detailed, for a better presentation of their similarities and differences (Figure S1).

Enzymes

The *Enzymes* tab of the main menu gives access to a list of classes and sub-classes of BLs, together with their corresponding BL families (Figure S3). This is represented as an *SVG* image that is dynamically generated from the raw data, which always ensures up-to-date information.

Each entry of a given BL family contains the class, protein name and eventually alternative names. When the family features several clearly defined sub-families, this information is also present. GenPeptID and GenBankID (with a RefSeq number when provided by NCBI) are also provided, with the corresponding links on the NCBI's website for more detailed information. Bibliographic

data (PubMedID, DOI), functional (phenotype, hydrolytic profile) and genetic (natural or acquired type) information and links to the other sections are also provided (Figure S2).

Sequence alignments are provided for each class, subclass, family and subfamily (Figure S4), together with the corresponding phylogenetic tree (Figure S5).

Structures

The *Structures* tab gives access to a table containing all three-dimensional structures of BLs reported in the Protein Data Bank¹⁴. Each entry contains the name of BL, together with the class or sub-class to which it belongs, followed by the PDB code and resolution (if applicable). The protein sequence is linked to the corresponding UniProt entry and, if appropriate, the existing mutations (extracted from the PDB file content) are shown. Bibliographic data (PubMedID, DOI) allows an easy retrieval of the original articles associated with the structure through links to PubMed and to the journal website. All ligands, buffer molecules and ions present in the structures are highlighted, together with their interaction mode with the protein (non-covalent, covalent, metal coordination). For all these molecules, links to their corresponding dedicated page on the PDB website are provided. Crystallographic details (space group, unit cell parameters, Z-value) are also presented, in order to facilitate the resolution of new structures and to allow an easy comparison of the existing ones (Figure S6).

Mutants

The synthetic mutants that were described for each enzyme in the literature are presented, together with bibliographical information (PubMedID, DOI) and links to PDB structures and hydrolytic profiles when appropriate (Figure S7). Given the very important number of synthetic mutants described to date, the present version of the BLDB is not complete. More mutants will be added in the near future.

Kinetics (hydrolytic profiles)

This section is organised in two parts: (i) a table containing the hydrolytic profiles on different β -lactam antibiotics, with values for the turnover number (k_{cat}), the Michaelis constant (K_m) and the catalytic efficiency (k_{cat}/K_m) (Figure S8); (ii) a radar chart representing a superposition of hydrolytic profiles selected for easier comparison (Figure S9). The number of hydrolytic profiles currently available in the BLDB is relatively modest, and more entries are scheduled to be added in the near future.

BLAST

Protein- and nucleotide-based BLAST search capabilities of BLDB are implemented using a personalised version of the SequenceServer graphical interface¹². The input sequence type (protein or nucleotide) is automatically detected, and the BLAST search type is adapted accordingly. Advanced parameters can be

used for the BLAST search in order to obtain more refined results (Figure S10).

The BLAST search is executed using the default parameters and the results are shown using a personalised interface, with the name of BL highlighted in red and links to the corresponding entries on the NCBI's website. The number of identical residues between the query and each sequence producing a significant alignment is provided, together with the percentage of identity (Figure S11). Together with the E-value, this represents useful information for a quick assessment of the BLAST results. A percentage of 100.00% means that the input sequence is already present in the BLDB, whereas a high sequence identity points out towards the BL class and/or family to which the input sequence might belong.

In the lower part of the results page, the alignments between the query and sequences producing significant alignments are provided (Figure S12).

Initial content

As of 25 April 2017, BLDB contains 2666 unique enzymes from all four classes of BLs, as well as 810 three-dimensional structures of BLs that are currently available in the Protein Data Bank (PDB)¹⁴. BLDB also contains 167 mutants and 47 hydrolytic profiles.

Conclusion

BLDB is developed and maintained by two well-established research groups that are active in the field of BL-mediated antibiotic resistance. This resource is designed to provide appropriate answers to the needs of the research and clinical communities working on antimicrobial resistance.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplementary Information

Beta-Lactamase DataBase (BLDB) – Structure and Function

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Beta-Lactamase DataBase - Structure and Function

Home

Enzymes

Structures

Mutants

Kinetics

BLAST



BLDB Presentation

Multidrug resistant (MDR) gram-negative pathogens, especially *Enterobacteriaceae*, are emerging worldwide. The MDR pattern is relatively common with resistance appearing to all major classes of anti-gram-negative agents (e.g. β -lactams, fluoroquinolones, and aminoglycosides), and in some cases, resistance to all available drugs. This is particularly worrisome in view of the current dearth of new compounds active against MDR gram-negatives in the pipeline.

β -Lactams, due to their safety, reliable killing properties and clinical efficacy, are among the most frequently prescribed antibiotics used to treat bacterial infections. However, their utility is being threatened by the worldwide proliferation of β -lactamases (BLs) with broad hydrolytic capabilities, especially in MDR gram-negative bacteria. These BLs are divided into 4 classes based on their sequence identities. Classes A, C and D contain active-site serine enzymes whose reaction pathways involve acylenzyme adducts while class B represents metallo- β -lactamases (MBLs) which do not form such intermediates (require zinc ion (s) for their function). Currently, BL-mediated resistance does not spare even the newest and most powerful β -lactams (i.e. carbapenems), whose activity is challenged by the MBLs (IMP, VIM, NDM, ...) as well as classes A and D serine-carbapenemases (KPC, IMI, GES, OXA-48, OXA-23, OXA-40, ...).

While a handful of β -lactamases were known in the early 1970's, the number of β -lactamases has ever since been growing rapidly, especially with novel enzymes described, and the current dissemination of some enzymes in clinical isolates that undergo changes in their amino-acid sequence, yielding novel hydrolytic properties. The substrate specificities may be relatively narrow or broad including the extended-spectrum cephalosporins and the carbapenems. The class A enzymes (known primarily as penicillinases) tend to hydrolyze penicillins over cephalosporins as substrates although many variants may hydrolyze significantly broad-spectrum cephalosporins and carbapenems. The class B enzymes (metallo- β -lactamases) typically have an extremely broad-spectrum substrate specificity including all β -lactams except monobactams (aztreonam). The class C enzymes (cephalosporinases) tend to prefer cephalosporins as substrates whereas class D enzymes (oxacillinases) have an unusually high substrate preference for oxacillin and related penicillins. None of the marketed β -lactam molecules may resist to the hydrolysis by β -lactamases.

The aim of the Beta-Lactamase DataBase (BLDB) is to compile sequence information as well as biochemical and structural informations on all the currently known β -lactamases. BLDB offers in addition tools to analyze β -lactamases and provides important links to the related web resources (NCBI, PDB, etc.). This comprehensive web-based database may provide at a glance useful insights in the structure-function relationships of β -lactamases, and thus allowing a better understanding of substrate specificities, determine key residues involved in substrate recognition and hydrolysis, and to foresee the impact of mutations in the hydrolysis profile.

Statistics

Enzymes:
Overall (2666); class A (1111); subclass B1 (202); subclass B2 (18); subclass B3 (60); class C (628); class D (564).

Structures:
Overall (810); class A (340); subclass B1 (161); subclass B2 (14); subclass B3 (45); class C (154); class D (96).

Mutants:
Overall (167); class A (91); subclass B1 (35); subclass B2 (3); subclass B3 (6); class C (19); class D (13).

Kinetics:
Overall (47); class A (21); subclass B1 (3); subclass B2 (0); subclass B3 (4); class C (0); class D (19).

Last updated: April 25, 2017.

The development of the BLDB database is funded in part by the [JPIAMR transnational project DesinMBL](#), the [Région Ile-de-France \(DIM Malin\)](#) and the [Laboratory of Excellence in Research on Medication and Innovative Therapeutics \(LERMIT\)](#).
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Contact: contact@bldb.eu

Fig. S1. Global overview of the Home page.

Enzymes

Ambler sub-class B1 beta-lactamases

Sequence alignment for sub-class B1

Ambler class	Protein name	Alternative protein names	Subfamily	GenPeptID	GenBankID	PubMedID (DOI)	Sequence	Number of PDB structures	Mutants	Phenotype	Functional information	Natural (N) or Acquired (A)
NDM												
The official nomenclature for NDM family is maintained at NCBI .												
B1	NDM-1			AHM26723	KJ018857	19770275 (DOI)	view	32	2	3a	Carbapenemase	A
B1	NDM-2			AE441876	JF703135	21427107 (DOI)	view			3a	Carbapenemase	A
B1	NDM-3			AFK80349	JQ734687	21330550 (DOI)	view	1		3a	Carbapenemase	A
B1	NDM-4			AFB82585	JQ348841	22252797 (DOI)	view	1		3a	Carbapenemase	A
B1	NDM-5			ANE23847	KX062216	21930874 (DOI)	view	1		3a	Carbapenemase	A
B1	NDM-6			AEX08599	JN967644	23731823 (DOI)	view	1		3a	Carbapenemase	A
B1	NDM-7			AKN32889	KP826705	23557929 (DOI)	view			3a	Carbapenemase	A
B1	NDM-8			AJ614442	KP265938	23459485 (DOI)	view	1		3a	Carbapenemase	A
B1	NDM-9			AGU91756	KC999080	24913967 (DOI)	view			3a	Carbapenemase	A
B1	NDM-10			AGT37351	KF361506		view			3a	Carbapenemase	A
B1	NDM-11			AJ614443	KP265939		view			3a	Carbapenemase	A
B1	NDM-12			BAO79439	AB926431	25092693 (DOI)	view			3a	Carbapenemase	A
B1	NDM-13			BAQ02518	LCB12596	26169399 (DOI)	view			3a	Carbapenemase	A
B1	NDM-14			AJP18054	KM210086	25645836 (DOI)	view			3a	Carbapenemase	A
B1	NDM-15			AKF43458	KP735848		view			3a	Carbapenemase	A
B1	NDM-16			AKZ20823	KP862821		view			3a	Carbapenemase	A
B1	NDM-17			AOT73359	KX812714		view			3a	Carbapenemase	A
B1	NDM-18			APZ75411	KY503030		view			3a	Carbapenemase	A
PEDO												
B1	PEDO-3			AJP77076	KP109679	26482314 (DOI)	view					N (Pedobacter sp. Stok-3)
SFB												

* after the protein name denotes a temporary name in BLDB, while waiting for a definitive assignment by NCBI. GeneBankIDs represented in bold face correspond to entries for which NCBI has assigned a RefSeq.

Fig. S2. Global overview of the Enzymes section.

Beta-Lactamase DataBase - Structure and Function											
Home		Enzymes		Structures		Mutants		Kinetics		BLAST	
Class A		Sub-class B1		Sub-class B2		Sub-class B3		Class C		Class D	
ACI	AER	BclI	BlaB	CphA	SFH	AIM	BJP	ACC	ACT	AmpH	BAC
AFA	AST	CGB	CfiA			CAR	CAU	ADC	AQU	BAD	BAT
Bcl	BCL	DIM	EBR			CPS	EAM	AsbA1	CepS	BED	BEN
BEL	BES	FIM	GIM			ECM	EFM	BUT	CAV	BOC	BPU
BIC	BKC	HMB	IMP			ELM	ESP	CepH	CFE	BSU	LCR
BlaS	BOR	IND	JOHN			EVM	FEZ	CHR	CMH	NPS	OXA
BPS	BRO	KHM	MOC			GOB	L1	CMY	DHA		
CAD	CARB	MUS	NDM			MSI	PEDO	Ear	EDC		
CblA	CBP	PEDO	SFB			POM	RM3	ERH	ESC		
CdiA	CepA	SIM	SLB			SMB	SPG	FOX	LHK		
CfxA	CGA	SPM	TMB			SPR	THIN	LYL	MIR		
CIA	CKO	TUS	VIM					MOR	MOX		
CME	CRH							OCH	PDC		
CRP	CRS							PLY	SEC		
CTX-M	DES							TRU	UCB		
ERP	FAR							XAN	YEC		
FEC	FONA							YER			
FPH	FRI										
FTU	GES										
GIL	GPA										
HugA	IMI										
KLUA	KLUC										
KLUG	KLUY										
KPC	L2										
LAP	LEN										
LUT	MAL										
MIN	OHIO										
OIH	OKP										
ORN	OXY										
PC1	PC2										
PER	PLA										
PME	PenA										
PenB	PenC										
PenD	PenE										
PenP	PSV										
RAHN	RCAP										
ROB	RUB										
SCA	SCO										
SED	SFC										
SFR	SFO										
SGM	SHV										
SME	TEM										
TER	TLA										
VCC	VEB										
VHH	VHW										
YENT											

Fig. S3. Global overview of the β -lactamase families that are present in the BLDB.

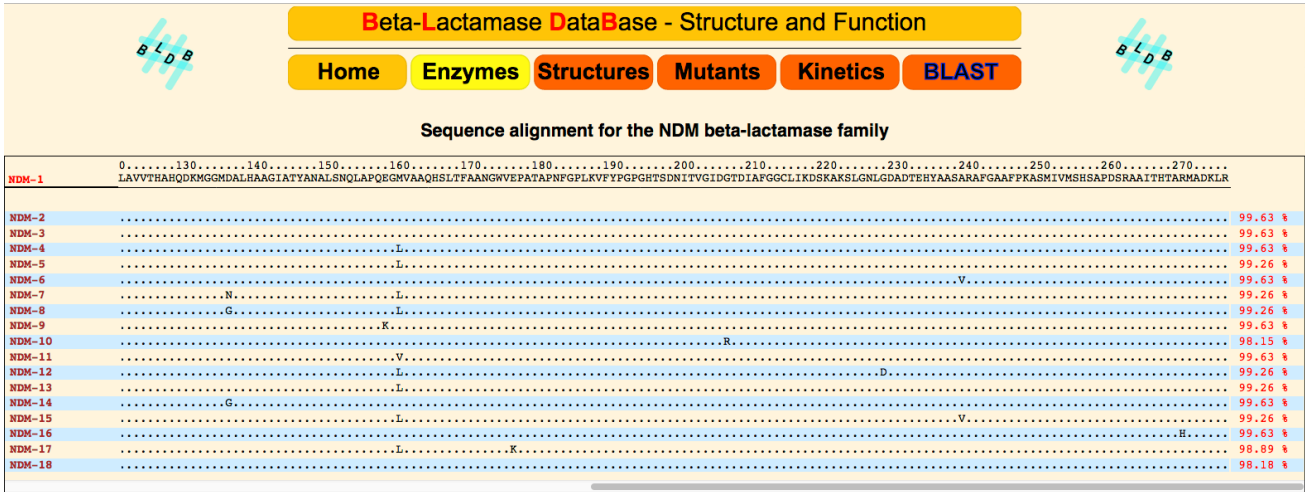


Fig. S4. Sequence alignment.

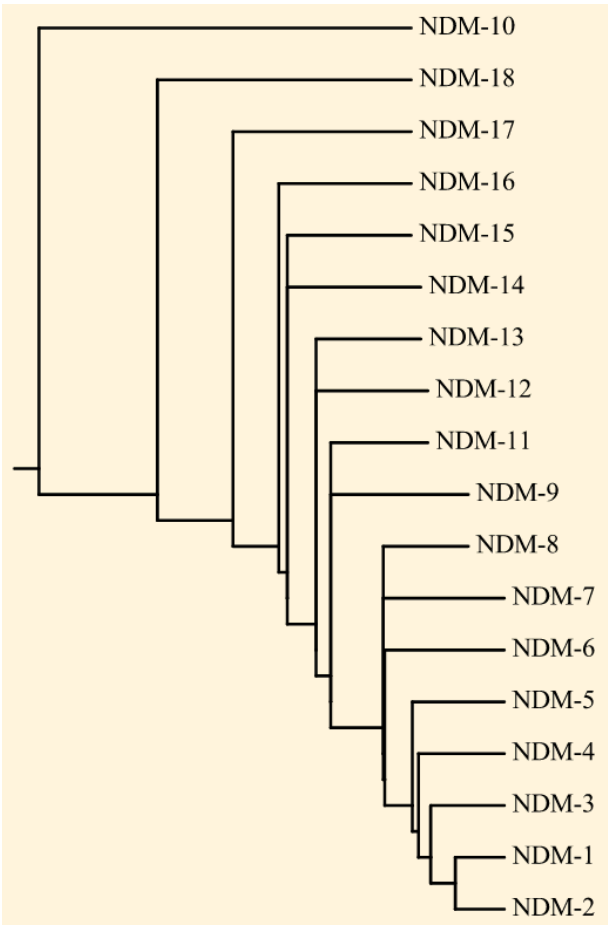


Fig. S5. Rooted phylogenetic tree corresponding to the sequence alignment.

Beta-Lactamase DataBase - Structure and Function

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Structures

Ambler class	Protein name	PDB code	Resolution (Å)	UniProt code	PubMed ID	DOI	PDB	Mutations	Ligands	Space group	Unit cell parameters	Z value
B1	NDM-1	3PG4	2.00	C7C422			ddp			I 4 2 2	77.539 77.539 177.740 • 90.00 90.00 90.00	16
B1	NDM-1	3Q6X	1.30	C7C422	21507902	10.1096/FJ.11-184036	ddp		*ZZZ GOL ZN	P 21 21 21	39.212 79.102 134.412 • 90.00 90.00 90.00	8
B1	NDM-1	3RKJ	2.00	C7C422	21931780	10.1371/JOURNAL.PONE.0024621	ddp		GOL SO4	P 1 21 1	59.712 51.113 70.680 • 90.00 106.96 90.00	4
B1	NDM-1	3RKK	2.35	C7C422	21931780	10.1371/JOURNAL.PONE.0024621	ddp		ACY GOL SO4	P 1 21 1	59.760 50.862 70.717 • 90.00 106.98 90.00	4
B1	NDM-1	3S0Z	2.50	ESK176	21637961	10.1007/S13238-011-1055-9	ddp		ZN	P 31	40.696 40.696 215.292 • 90.00 90.00 120.00	6
B1	NDM-1	3SBL	2.31	C7C422	21931780	10.1371/JOURNAL.PONE.0024621	ddp		ClT	I 2 2 2	66.050 83.269 105.401 • 90.00 90.00 90.00	8
B1	NDM-1	3SFP	2.27	C7C422	21931780	10.1371/JOURNAL.PONE.0024621	ddp		ClT Cl GOL SO4 ZN	P 43 21 2	97.944 97.944 187.547 • 90.00 90.00 90.00	32
B1	NDM-1	3SPU	2.10	C7C422	21774017	10.1002/PRO.697	ddp		ZN	P 1	66.536 73.904 77.407 • 70.32 75.86 65.30	5
B1	NDM-1	3SRX	2.50	C7C422			ddp	Q37N Q38Y	CD Cl GOL SCN	P 61 2 2	108.945 108.945 237.883 • 90.00 90.00 120.00	24
B1	NDM-1	3R89	1.91	C7C422	22102018	10.1107/S174430911029654	ddp		CD Cl Ni ZN	I 4 2 2	120.300 120.300 88.100 • 90.00 90.00 90.00	16
B1	NDM-1	4EXS	2.40	C7C422	22713171	10.1021/JA303579D	ddp		*XHZ ZN	P 41 21 2	107.120 107.120 92.900 • 90.00 90.00 90.00	16
B1	NDM-1	4EXT	1.47	C7C422	22713171	10.1021/JA303579D	ddp		EDQ ZN	P 41 21 2	107.220 107.220 92.850 • 90.00 90.00 90.00	16
B1	NDM-1	4ETZ	1.17	C7C422	22713171	10.1021/JA303579D	ddp		*DMZ ZN	P 21 21 21	39.120 79.360 133.830 • 90.00 90.00 90.00	8
B1	NDM-1	4EVB	1.16	C7C422	22713171	10.1021/JA303579D	ddp		*DMQ ZN	P 21 21 21	39.200 79.370 134.150 • 90.00 90.00 90.00	8
B1	NDM-1	4EYF	1.80	C7C422	22713171	10.1021/JA303579D	ddp		*DMG ZN	P 21 21 21	39.030 79.490 134.210 • 90.00 90.00 90.00	8
B1	NDM-1	4EYL	1.90	C7C422	22713171	10.1021/JA303579D	ddp		*DMV ZN	P 41 21 2	106.000 106.000 92.580 • 90.00 90.00 90.00	16
B1	NDM-1	4GYQ	1.35	C7C422			ddp	D223A	EDQ MG	P 1 21 1	69.140 74.225 76.595 • 90.00 90.11 90.00	8
B1	NDM-1	4GYU	1.80	C7C422			ddp	A121F	GOL IMD PEG PEG	P 65	101.473 101.473 42.679 • 90.00 90.00 120.00	6
B1	NDM-1	4H0D	1.50	C7C422	23363572	10.1096/fi.12-224014	ddp		*ZZZ EDQ FMT GOL MN NA SO4	P 21 21 21	39.101 78.606 133.711 • 90.00 90.00 90.00	8
B1	NDM-1	4HKY	2.00	C7C422	23363572	10.1096/fi.12-224014	ddp		*FPM *SFR CD Cl GOL	C 1 2 1	145.627 39.324 75.370 • 90.00 99.96 90.00	8
B1	NDM-1	4HLJ	1.50	C7C422	23363572	10.1096/fi.12-224014	ddp		*ZZZ CD Cl	P 21 21 21	38.997 78.700 134.377 • 90.00 90.00 90.00	8
B1	NDM-1	4HLZ	1.05	C7C422	23363572	10.1096/fi.12-224014	ddp		*ZZZ EDQ ZN	P 21 21 21	39.190 79.171 134.473 • 90.00 90.00 90.00	8
B1	NDM-1	4RAM	1.50	C7C422			ddp	M67V	*PNM Cl ZN	P 21 21 21	39.065 79.212 133.307 • 90.00 90.00 90.00	8
B1	NDM-1	4RAW	1.30	C7C422			ddp	M67V	*ZZZ CD EDQ	P 21 21 21	39.114 78.769 133.815 • 90.00 90.00 90.00	8
B1	NDM-1	4RBS	2.40	C7C422			ddp		*BRV ACY GOL ZN	P 31 2 1	74.428 74.428 219.960 • 90.00 90.00 120.00	12

Legend for ligands: * Ligand covalently-bound to active site residues; S Non-covalent ligand (Michaelis complex); # Ligand coordinated to active site metal ions.

Fig. S6. Global overview of the Structures section.

Beta-Lactamase DataBase - Structure and Function

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[Structures](#)
[Mutants](#)
[Kinetics](#)
[BLAST](#)

Mutants

Ambler class	Protein name	Mutations	PubMed ID	DOI	PDB structures	Hydrolytic profile
A	CTX-M-9	Q25PCA	19305397	10.1038/NCHEMBIO.155	view	
A	CTX-M-9	S70G	20452359	10.1016/J.JMB.2010.04.062	view	
A	CTX-M-9	S70G	21930882	10.1128/AAC.00245-11	view	
A	KPC-2	C-ter 292VNCQ296 deleted	18625772	10.1128/AAC.00163-08		
A	KPC-2	G175S	19731932	10.1021/BI9007963	view	
A	KPC-2	W105F	20620006	10.1002/pro.454		view
A	KPC-2	W105N	20620006	10.1002/pro.454		view
A	KPC-2	W105L	20620006	10.1002/pro.454		view
A	KPC-2	W105V	20620006	10.1002/pro.454		view
A	KPC-2	T237S	20421396	10.1128/AAC.00197-10	view	
A	KPC-2	T237A	20421396	10.1128/AAC.00197-10	view	
A	KPC-2	R220H	22687511	10.1128/AAC.05769-11	view	
A	KPC-2	R220A	22687511	10.1128/AAC.05769-11	view	
A	KPC-2	R220M	22687511	10.1128/AAC.05769-11	view	
A	KPC-2	R220K	22687511	10.1128/AAC.05769-11	view	
A	KPC-2	E276A	22687511	10.1128/AAC.05769-11	view	
A	KPC-2	E276D	22687511	10.1128/AAC.05769-11	view	
A	PC1-1	A238S I239DEL	8869640	10.1093/PROTEIN/8.12.1275		
A	PC1-1	N170D E166Q	10436083	10.1093/PROTEIN/12.7.573		
A	PC1-1	N170Q	8987980	10.1021/BI962242A	view	
A	PC1-1	N170M	8987980	10.1021/BI962242A	view	
A	PC1-1	G179N	1892849	10.1021/BI001034017	view	
A	PC1-1	E166D N170Q	11327855	10.1021/BI002277H		
A	PC1-1	K73H	8823158	10.1021/BI961153V	view	
A	PC1-1	S70A	8823158	10.1021/BI961153V	view	
A	PenP	E166A	8433965	10.1093/PROTEIN/6.1.11	view	
A	PenP	E166C	21705325	10.1074/JBC.M110.198895	view	

Fig. S7. Global overview of the Mutants section.

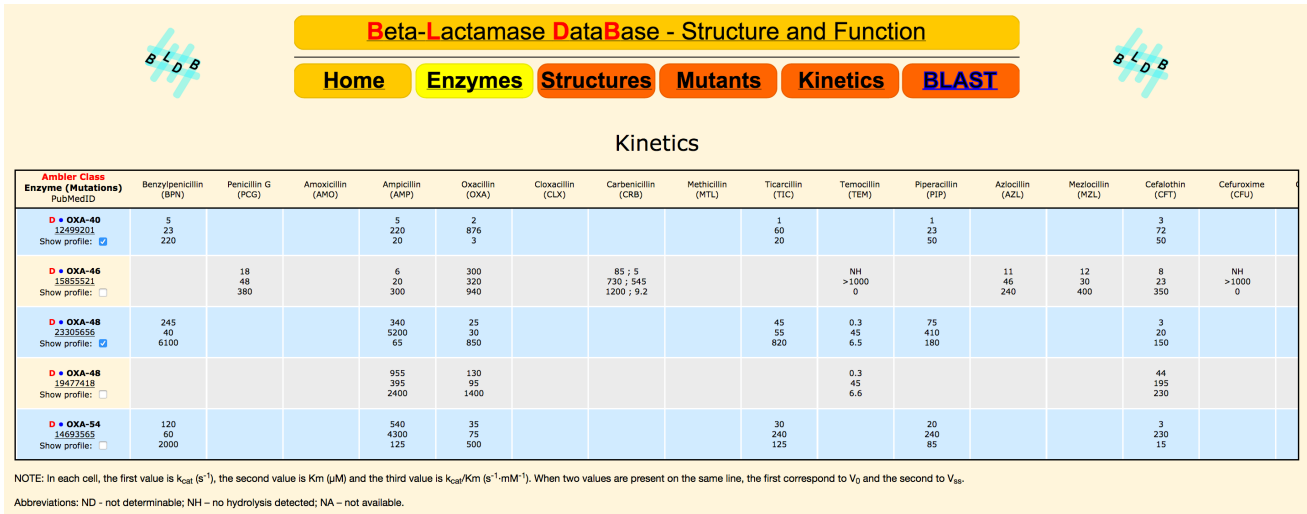


Fig. S8. Global overview of the Kinetics section, showing the hydrolytic profiles.

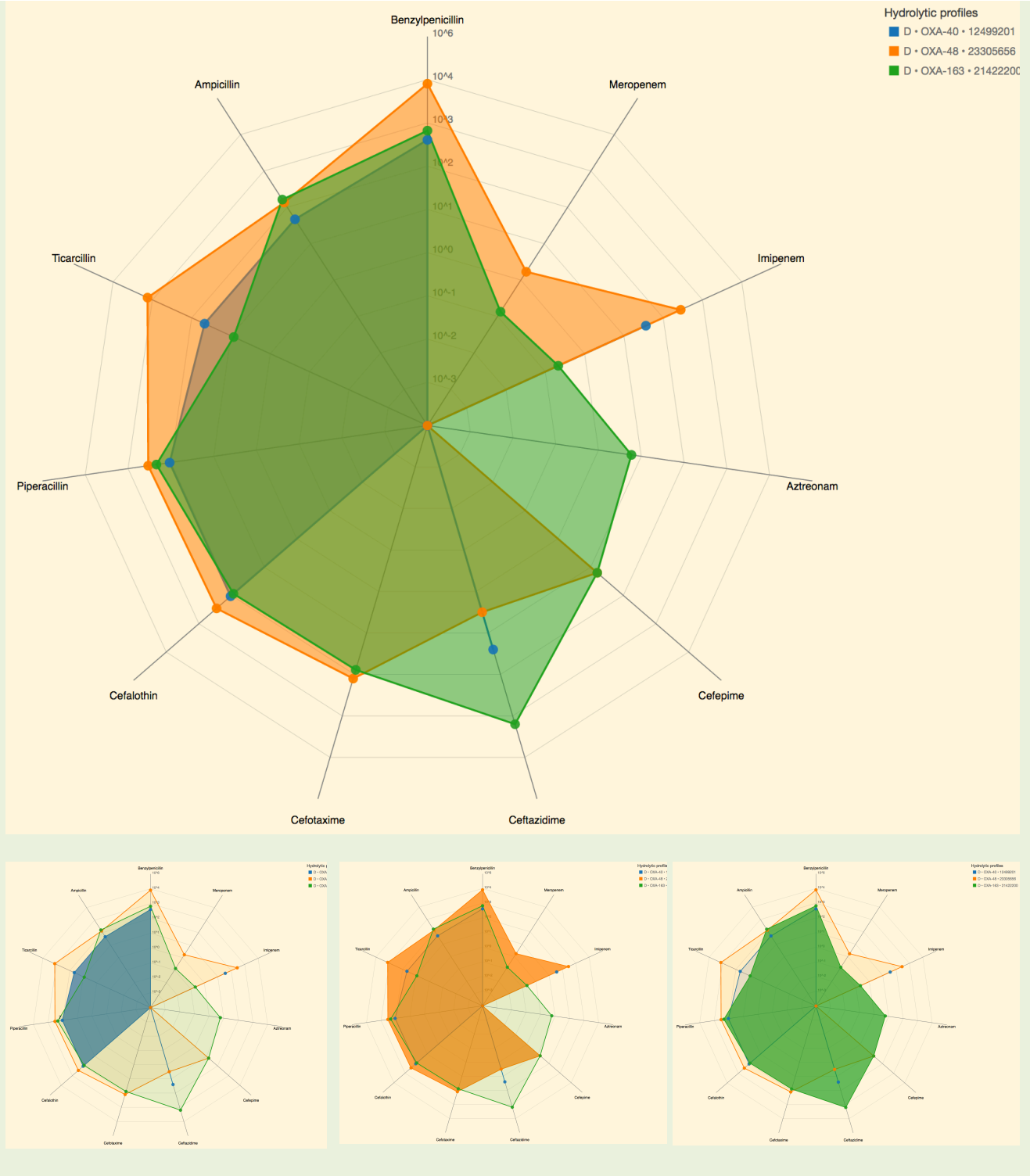


Fig. S9. Radar chart showing the superposition of hydrolytic profiles for OXA-40 (blue), OXA-48 (orange) and OXA-163 (green).

SequenceServer 1.0.8

Help & Support

>gi|595266892|gb|AHM26723.1|NDM-1| beta-lactamase NDM-1 [Achromobacter sp. NF518]
MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDRFGDLVFRQLAPNVWQHTSYLDMPGF
GAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWIQEIINLPVALAVVTHAQDKMGMDALHAAGIATY
ANALSNQLAPQEGMVAAQHSLTFAANGWVEPATPNFGPLKVFYPGPGHTSDNITVGIDGTDIAFGGCLI
KDSKAKSLGNLGDADTEHYAASARAFGAAPKASMIVMSHSAFDSRAAITHTARMADKLR

Detected: protein sequence(s).

Nucleotide databases

☐ beta-lactamases-all-nucleotide

Protein databases

☒ beta-lactamases-all-protein

Advanced Parameters: eg: -evalue 1.0e-5 -num_alignments 100

BLASTP

Fig. S10. SequenceServer graphical interface for the nucleotide- and protein-based BLAST queries.

– S8 –

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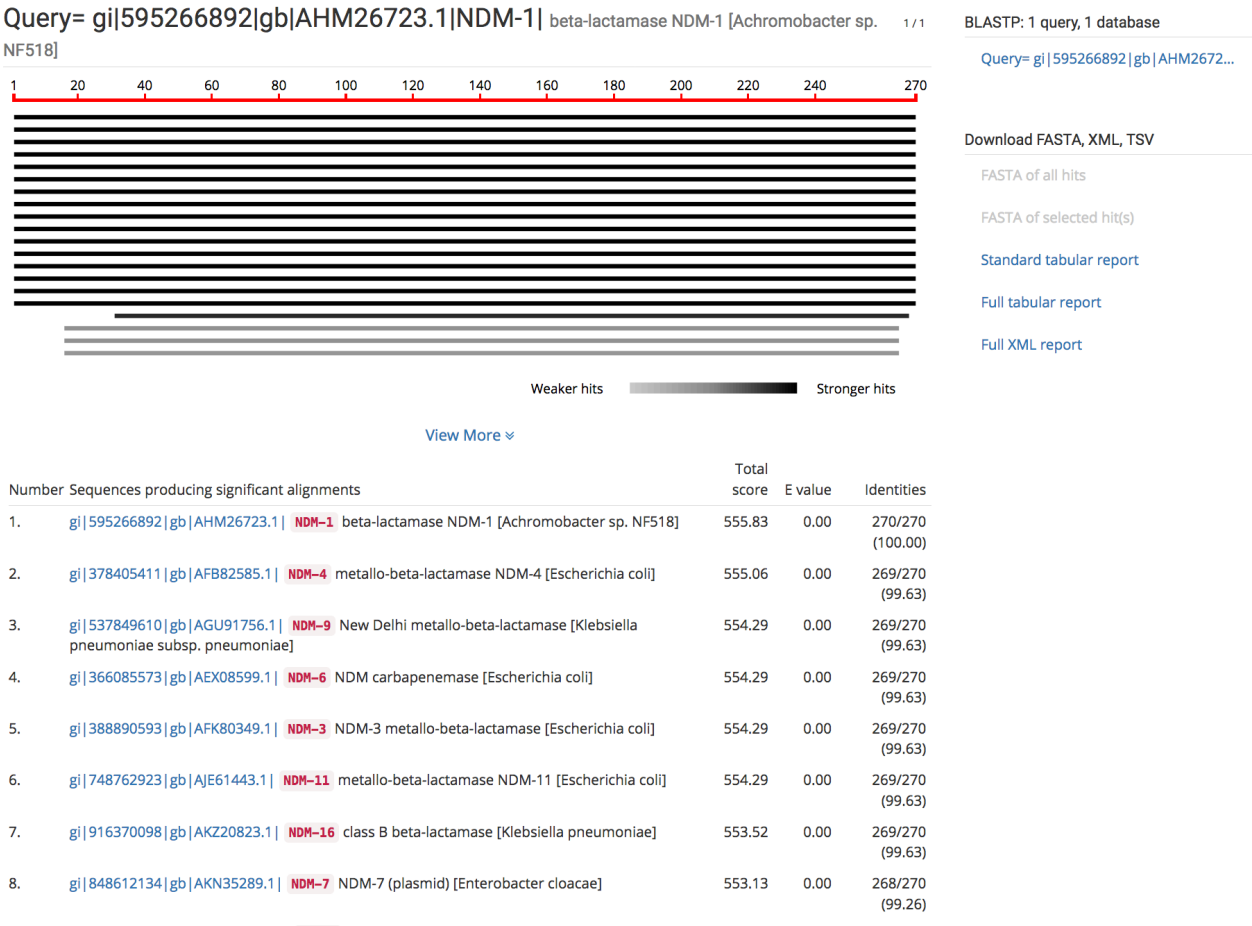


Fig. S11. SequenceServer formatted results of a protein BLAST query.

▼ gij595266892|gb|AHM26723.1| #NDM-1# beta-lactamase NDM-1 [Achromobacter sp. NF518]

1 / 299

Hit length: 270

Select

Sequence

FASTA

NCBI

1. Positives	Score	Identities	Gaps	E value
270/270 (100.00)	555.83 (1431)	270/270 (100.00)	0/270 (0.00)	0.00 × 10 ⁺⁰⁰
Query 1	MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETG			60
Subject 1	MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETG			60
Query 61	HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWKQEI			120
Subject 61	HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWKQEI			120
Query 121	AHQDKMGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL			180
Subject 121	AHQDKMGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL			180
Query 181	KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAAF			240
Subject 181	KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAAF			240
Query 241	PKASMIVMSHSAPDSRAAITHTARMADKLR	270		
Subject 241	PKASMIVMSHSAPDSRAAITHTARMADKLR	270		

▼ gij378405411|gb|AFB82585.1| #NDM-4# metallo-beta-lactamase NDM-4 [Escherichia coli]

2 / 299

Hit length: 270

Select

Sequence

FASTA

NCBI

1. Positives	Score	Identities	Gaps	E value
270/270 (100.00)	555.06 (1429)	269/270 (99.63)	0/270 (0.00)	0.00 × 10 ⁺⁰⁰
Query 1	MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETG			60
Subject 1	MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETG			60
Query 61	HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWKQEI			120
Subject 61	HTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILNWKQEI			120
Query 121	AHQDKMGMDALHAAGIATYANALSNQLAPQEGMVAAQHSLTFAANGWVEPATAPNFGPL			180
Subject 121	AHQDKMGMDALHAAGIATYANALSNQLAPQEG+VAAQHSLTFAANGWVEPATAPNFGPL			180
Query 181	KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAAF			240
Subject 181	KVFYPGPGHTSDNITVGIDGTDIAFGGCLIKDSKAKSLGNLGDADTEHYAASARAFGAAAF			240
Query 241	PKASMIVMSHSAPDSRAAITHTARMADKLR	270		
Subject 241	PKASMIVMSHSAPDSRAAITHTARMADKLR	270		

BLASTP: 1 query, 1 database

Query= gij595266892|gb|AHM26723.1...

Download FASTA, XML, TSV

FASTA of all hits

FASTA of selected hit(s)

Standard tabular report

Full tabular report

Full XML report

Fig. S12. Sequence alignment of protein BLAST results with the initial query.

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A GREATER THAN EXPECTED VARIABILITY AMONG OXA-48-LIKE CARBAPENEMASES

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ABSTRACT

Background: OXA-48-like carbapenemases represent a major health concern given their difficult detection, their epidemic behavior and their propensity to modify their spectrum of hydrolysis through point mutations.

Objective: To get an extensive view on the current variability among OXA-48-like enzymes, we have retrieved all the sequences available from NCBI (National Center for Biotechnology Information).

Method: We carried out several BLAST (Basic Local Alignment Search Tool) searches in the NCBI's "nr" and "nr_env" databases (downloaded on December 20th, 2016) using known members of OXA-48-like subfamily as query.

Results: While 23 variants have assigned OXA-numbers, 62 novel alleles have been identified. They correspond to novel enzymes with mutations located in some cases within the conserved active site motives. The important number of novel variants identified by this study is of great interest, since it provides a more realistic assessment of OXA-48-like variants.

Conclusion: A large variety of OXA-48-like enzymes has been unraveled through our bioinformatic search for variants. The finding of OXA-48-like enzymes in environmental isolates may reflect the contamination by Enterobacteriaceae producing OXA-48-like enzymes and/or the presence of *Shewanella* spp. isolates.

Keywords: OXA-48, variability, variants.

REZUMAT

Introducere: Carbapenemazele OXA-48-like reprezintă o problemă majoră pentru sănătate, având în vedere detectarea lor dificilă, comportamentul epidemic și tendința lor de a-și modifica spectrul de hidroliză prin mutații punctiforme.

Obiectiv: Pentru a obține o imagine amplă asupra variabilității actuale a enzimelor OXA-48-like, am recuperat toate secvențele disponibile de la NCBI (National Center for Biotechnology Information).

Metodă: Am efectuat mai multe căutări BLAST (Basic Local Alignment Search Tool) în bazele de date „nr” și „nr_env” ale NCBI (descărcate pe 20 decembrie 2016), utilizând ca interogare membrii cunoscuți ai sub-familiei OXA-48.

Rezultate: În timp ce unui număr de 23 de variante li s-au atribuit numere OXA, au fost identificate 62 de alele noi. Acestea corespund noilor enzime cu mutații localizate în unele cazuri în cadrul motivelor conservate ale site-ului activ. Numărul important de variante noi identificate în acest studiu este de mare interes, deoarece oferă o evaluare mai realistă a variantelor de tip OXA-48-like.

Concluzie: O mare varietate de enzime OXA-48-like a fost descoperită în urma căutării bioinformatică a variantelor. Detectarea enzimelor OXA-48-like în izolate din mediu poate reflecta contaminarea cu Enterobacteriaceae producătoare de enzime OXA-48-like și/sau prezența de izolate *Shewanella* spp.

Cuvinte-cheie: OXA-48, variabilitate, variante.

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INTRODUCTION

In the last decade, the emergence of carbapenem-resistance in Gram-negatives has been observed worldwide, both in non-fermenters and in *Enterobacteriaceae* [1, 2]. The emergence of carbapenemase-producing *Enterobacteriaceae* (CPE) has become a major public health concern [1, 3]. Among these CPEs, OXA-48-producing *Enterobacteriaceae* have now widely disseminated throughout European countries and are identified on all continents [3, 4]. OXA-48 confers high-level resistance to penicillins, including temocillin, and hydrolyzes carbapenems at a low level, but spares extended-spectrum cephalosporins [5, 6]. OXA-48-like enzymes are Ambler class D enzymes, that belong to active-serine β -lactamases. According to the DBL (class D β -lactamase numbering scheme), oxacillinases possess a serine residue at position DBL 70 and a carbamoylated lysine at position DBL 73 [5, 7, 8]. The YGN (positions 144 to 146) and KTG (positions 216 to 218) motives are mostly conserved in oxacillinase sequences, the YGN motif being replaced by a FGN motif in several cases [5, 9]. The omega loop plays an interesting role in the function of the beta-lactamases: mutations in the “omega loop region” of a beta-lactamase can change its specific function and substrate profile, perhaps due to an important functional role of the correlated dynamics of the region [5]. A sign of the current spread of OXA-48-like enzymes is the identification of point mutant derivatives, differing by few amino acid substitutions or deletions. Most of these differences are located within the β 5- β 6 loop, which is important in the substrate specificity of OXA-48 [9-11].

Whereas some OXA-48-variants (mostly point mutant derivatives) have similar hydrolytic activities as compared to OXA-48 (OXA-181, OXA-204), others have slightly increased carbapenem-hydrolyzing activities (OXA-162) or slightly reduced carbapenem and temocillin hydrolyzing activities (OXA-232, OXA-244) [11, 12]. In contrast, variants presenting a four amino acid deletion within the β 5- β 6 loop (Table 1) such as OXA-163, OXA-247, OXA-405 have lost their carbapenem-hydrolytic activity but gained instead the capacity to hydrolyze expanded-

spectrum cephalosporins (Fig. 1, Table S1) [11, 13]. A comprehensive list of variants identified in clinical isolates can be found in the Beta-Lactamase DataBase (<http://bldb.eu/alignment.php?align=D:OXA-48-like>).

Twenty-two OXA-48 variants have been described, most of them from enterobacterial isolates, and have been assigned an OXA-number initially by Lahey Clinic (<http://www.lahey.org/Studies/other.asp>), and now by the Bacterial Antimicrobial Resistance Reference Gene Database at NCBI (<https://www.ncbi.nlm.nih.gov/bioproject/313047>). *Shewanella* species have been suggested as the reservoir of *bla*_{OXA-48} type oxacillinase genes [14]. This is the case for OXA-54 from *S. oneidensis* as it shares significant amino acid sequence identity with OXA-48. *Shewanella xianmensis* has recently been identified as the progenitor of OXA-181, OXA-48, and OXA-204 [15, 16]. However, this is not the case for all *Shewanella* species, since *S. algae* produces OXA-55, which has only 57% sequence identity with OXA-48 [14]. Several natural variants (7/23) have been described in *Shewanella* sp. isolates and have been assigned an OXA number. For these variants, kinetic data are not always available [7].

With the high throughput sequencing of many bacterial genomes, and the tremendous amount of metagenomic sequencing data, the available bacterial DNA sequences increase exponentially in the databases. In many cases, these sequence entries have not been carefully analyzed in respect to β -lactamase gene content. In this study, we have searched for the presence of OXA-48-like enzymes in these genomic and metagenomic sequence databases.

MATERIAL AND METHODS

We carried out several BLAST searches in the NCBI's “nr” and “nr_env” databases (downloaded on December 20th, 2016) using known members of OXA-48-like subfamily as query.

ClustalW was used to align the protein sequences of the identified novel chromosomally- and plasmid-encoded OXA-48-like β -lactamases with those already published, and Dendroscope was used to construct a phylogram [17, 18]. The references of all these enzymes can be found in the Beta-

Table 1. Sequence alignment for the OXA-48-like subfamily of class D β -lactamases. Only the sequences and the positions with mutations or deletions from loop β 5- β 6 are shown. The complete alignment can be found in the Beta-Lactamase DataBase at <http://bldb.eu/alignment.php?align=D:OXA-48-like> [7]

DBL ^a AA numbering scheme	219 ^c	220	224	225	226	227	228	229	230
OXA-48 AA numbering ^b	211	212	213	214	215	216	217	218	219
OXA-48	Y	S	T	R	I	E	P	K	I
OXA-54								Q	
OXA-162			A						
OXA-163, OXA-439		— ^d	—	—	—	D	T		
OXA-232				S					
OXA-244, OXA-484				G					
OXA-247	S	—	—	—	—	N	T		
OXA-370		E							
OXA-405		—	—	—	—	S			
OXA-436			V						
OXA-438		G	Y	—	—	D	T		
OXA-538			G						F
OXA-D281, OXA-D282, OXA-D284			V						
OXA-D303	C								
OXA-D312		P							
OXA-D340					V				

^a Class D β -lactamase numbering scheme [5, 8];

^b OXA-48 specific numbers. Numbers in bold correspond to residues of the β 5- β 6 loop ;

^c Only the sequences and the positions with mutations or deletions from loop β 5- β 6 are shown. The complete alignment can be found in the Beta-Lactamase DataBase at <http://bldb.eu/alignment.php?align=D:OXA-48-like>

^d — indicates a deletion.

Lactamase DataBase at <http://bldb.eu/BLDB.php?class=D#OXA> [7]; (b) Sequence of OXA-48; (c) Three-dimensional structure of OXA-48 (PDB 3HBR). Active site serine 70 is colored in green, and the residues from loop β 5- β 6 are colored with different shades of blue.

RESULTS-DISCUSSION

In this way, we could identify, along with the known 23 members of the OXA-48-like subfamily (enzymes with an assigned OXA-name), 62 novel OXA-48-like variants (displaying at least one point mutation to any of the 23 known variants) belonging to the OXA-48-like subfamily, and thus that are not present in the Bacterial Antimicrobial Resistance Reference Gene Database (<https://www.ncbi.nlm.nih.gov/bioproject/313047>).

No OXA-number has been assigned to these variants, since most of them were not isolated in clinical settings and their identification was primarily based on *in silico* analysis. These variants have been added to the BLDB database, as OXA-D type enzymes followed by a number, while waiting for a definitive assignment by NCBI. These novel variants show >90% sequence identity as compared with OXA-48. The nucleotide and protein sequences along with their GenBank accession numbers can be found in the Beta-Lactamase DataBase (<http://bldb.eu/BLDB.php?class=D#OXA>) [7].

Among the sequences presented in Table S1, 2 were retrieved from dye-degrading bacteria [17, 18], 7 from *Shewanella* sp., and 43 from uncultured bacteria.

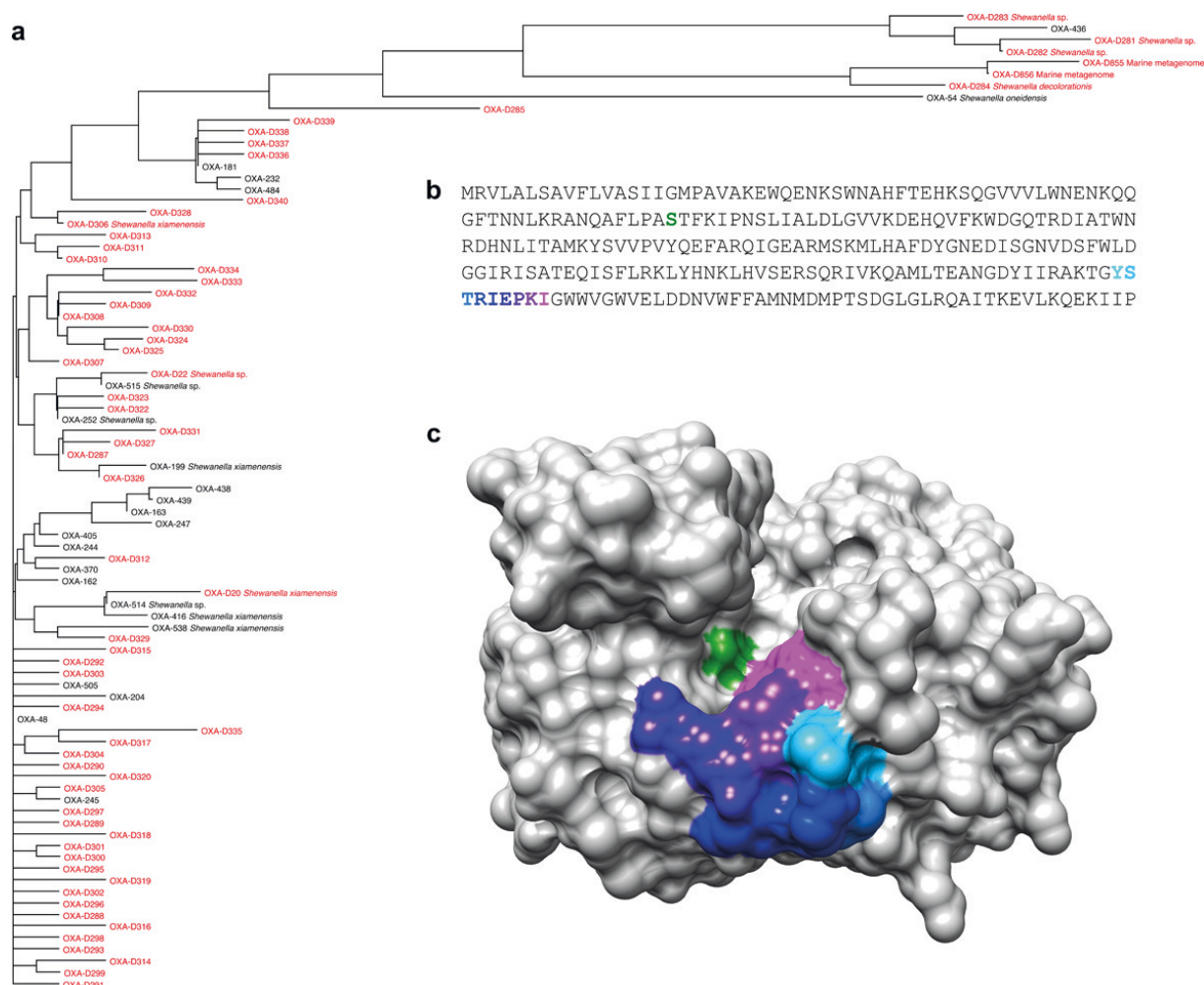


Fig. 1. (a) Phylogeny of the chromosomally- and plasmid-encoded class D β -lactamases belonging to the OXA-48-like subfamily. For naturally-occurring enzymes, the bacterial host name is indicated. The alleles identified in this study are colored in red. The phylogram was constructed with Dendroscope using ClustalW aligned protein sequences [19, 20]. The references of all these enzymes can be found in the Beta-Lactamase DataBase at <http://bldb.eu/BLDB.php?class=D#OXA> [7]; **(b)** Sequence of OXA-48; **(c)** Three-dimensional structure of OXA-48 (PDB 3HBR). Active site serine 70 is colored in green, and the residues from loop β 5- β 6 are colored with different shades of blue.

Among the 62 novel variants a great variability has been observed differing by one up to 24 amino-acids (Fig. 1). The most distantly related variants are close to OXA-436, a carbapenemase responsible of an outbreak in Denmark. The amino acid changes are scattered all over the sequence, but some are located within the conserved regions of class D enzymes (represented as boxes in the Supplementary Information file), and thus are likely to generate altered phenotypes. For instance, the F72I change in OXA-D320, which is located next to the S70 and the K73 may impact the activity of the enzyme. Similarly, Y144C and N146S, found in OXA-D319 and

D338, respectively, are located within the YGN motif. A Y to F change is responsible of NaCl resistance of oxacillinases, as shown for OXA-40 [9].

The T217A change is located within the highly conserved KTG box. This threonine has been replaced by serine in OXA-40 without effect on hydrolysis, but the effect of an alanine in this position has not been addressed [9].

Finally, three novel variants were found with changes in the β 5- β 6 loop. The T221V as found in OXA-D281, -D282, and -D284 may have increased hydrolysis as shown for OXA-162, even though in the latter the T was replaced by A [11]. The S220P (OXA-D312)

may alter significantly the loop conformation, which would likely induce a drastic effect on the activity of the enzyme. The I226V is likely silent in terms of activity, as we have recently shown that I226A replacement does not alter the activity of the enzyme (T. Naas, personal communication).

CONCLUSION

Genomic and metagenomics data turn to be an inestimable source for discovering novel resistance gene or derivatives of known genes. In most cases, the reasons these genomic or metagenomics data were generated were not linked to antibiotic resistance studies. A large variety of OXA-48-like enzymes has been unraveled through our bioinformatic search for variants. The finding of OXA-48-like enzymes in environmental isolates may reflect the contamination by Enterobacteriaceae producing OXA-48-like enzymes and/or the presence of *Shewanella* spp. isolates. The important number of novel variants identified by this study is of great interest, since it provides a more realistic assessment of OXA-48-like variants. The finding of some amino-acid changes in the key boxes or the β 5- β 6 loop, suggests likely changes in hydrolysis profile. Further work will be necessary to address these issues.

Acknowledgements

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Conflict of interests: None to declare.

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Section 3: Participation to the D3R Grand Challenge 2 and development of innovative molecular modelling protocols

The Drug Design Data Resource (D3R, <https://drugdesigndata.org/>) regularly organizes blinded prediction challenges with the aim to evaluate the performance of tools and protocols that are used in real-life computer-aided drug discovery projects. The D3R Grand Challenge 2, held in 2016, was focused on a single protein, the farnesoid X receptor. In phase 1 the participants were asked to provide affinity predictions for 102 FXR ligands and pose predictions for 36 of them. Upon release of additional structural data participants were asked in phase 2 to repeat the affinity predictions. The challenge serves to test and improve the *in silico* methods the we apply in the laboratory, and compare our performance to that of other laboratories. Our participation to the D3R Grand Challenge 2 involved a protocol in two steps, the first step consists in a preliminary analysis of information available in literature (structural, and in some cases enzymatic data), which allows the identification of the best combination of docking software and scoring function that are suited for studying the system of interest. In the second step, the combination of docking software and scoring function is used to predict the binding modes (pose prediction) and the relative affinities of ligands (scoring).

All these methods are usually applied in our laboratory on daily basis, and have been used for the molecular modelling studies described in this manuscript.

Blinded evaluation of farnesoid X receptor (FXR) ligands binding using molecular docking and free energy calculations

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Abstract Our participation to the D3R Grand Challenge 2 involved a protocol in two steps, with an initial analysis of the available structural data from the PDB allowing the selection of the most appropriate combination of docking software and scoring function. Subsequent docking calculations showed that the pose prediction can be carried out with a certain precision, but this is dependent on the specific nature of the ligands. The correct ranking of docking poses is still a problem and cannot be successful in the absence of good pose predictions. Our free energy calculations on two different subsets provided contrasted results, which might have the origin in non-optimal force field parameters associated with the sulfonamide chemical moiety.

Keywords Docking · Scoring function · Gold · Vina · Autodock · Farnesoid X receptor · FXR · D3R · Drug design data resource · Grand Challenge 2

Introduction

Drug Design Data Resource (D3R, <https://drugdesign-data.org/>) organizes, on a regular basis, blinded prediction

challenges with the aim to evaluate the performance of tools and protocols that are used in real-life computer-aided drug discovery projects. To achieve this, datasets presenting different levels of difficulty are presented to the community, which is asked to predict, in “blind” conditions, the binding modes and the relative affinities of compounds.

The D3R Grand Challenge 2, which was held in 2016, was focused on a single protein, farnesoid X receptor (FXR, Fig. 1), a target with multiple potential applications that has received much attention during the recent years [1–30].

In Phase 1 the participants were asked to provide affinity predictions for 102 FXR ligands and pose predictions for 36 of them. In Phase 2 the participants were required to provide the same affinity predictions as in Phase 1, taking into account the additional structural data (36 new protein–ligand complexes) released at the end of Phase 1.

Figure 2 shows the chemical structures of compounds from FXR dataset for which the pose predictions were required. Most of the compounds included in this dataset can be organized in four homogeneous classes based on their chemical structures (benzimidazoles, isoxazoles, sulfonamides, spiro compounds), and the remaining ones presented inhomogeneous structures and were included in a group called miscellaneous. Biological activities data were available for some compounds from this dataset [31–33]. The exact composition of each group can be found in the Electronic Supplementary Material, as well as the structures of the entire FXR dataset, containing 102 ligands used for ranking prediction (Figure S1).

Additionally, the participants were asked to predict the relative affinities for two homogeneous subsets of compounds that are suited for free energy calculations. The structures of compounds (count of 15 and 18, respectively) included in the two free energy subsets are presented in Figs. 3 and 4.

Edithe Selwa, Eddy Elisée and Agustin Zavala have contributed equally to this work.

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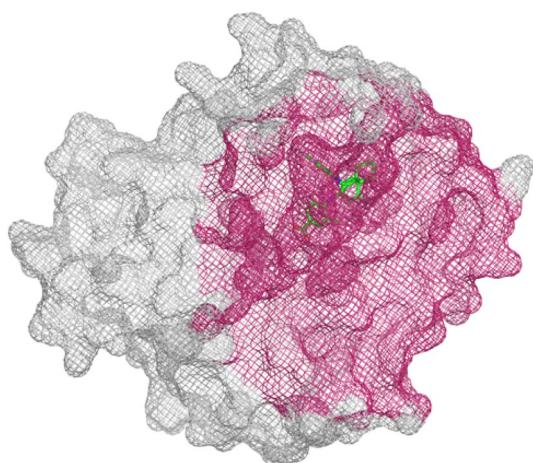


Fig. 1 Mesh surface representation of a representative crystal structure (PDB code 3OLF) of the FXR target. The binding site, as defined for our docking studies, is colored in *red*, and the ligand is colored in *green*

Methods

Protein structures

We found 27 crystal structures available in the Protein Data Bank (PDB) [34] for FXR (see the Electronic Supplementary Material for the complete list). These structures constituted our evaluation dataset. All ligands, ions and solvent molecules that were present were manually removed, then the structures were superimposed on the reference structure apo FXR provided by the D3R Grand Challenge organizers, in order to conserve the same coordinate system through the whole process. Missing residues in the structures were added using Modeller 9v12 [35]. Hydrogen atoms were added using Hermes, the graphical interface of Gold v5.2.2 [36] software, or with AutoDock Tools [37] prior to docking.

Ligands

Ligand structures from the evaluation dataset were retrieved from PDB in the SMILES format and they were converted into three-dimensional MOL2 files using CORINA v3.60 (<http://www.molecular-networks.com/>). This protocol was used instead of retrieving directly the three-dimensional coordinates from the PDB in order to avoid any bias in the docking process that might be related to the initial coordinates of the ligands. Ligand structures used in Phase 1 were obtained from the SMILES strings provided by organizers upon conversion into three-dimensional MOL2 files using CORINA. Three-dimensional coordinates of the ligands used in Phase 2 were built using UCSF Chimera [38], by superimposing their common backbone on the released FXR_17, FXR_10 and FXR_12 crystal structures and by

manual addition of the appropriate substituents. In all cases, the protonation state for all compounds was adjusted at physiological pH using LigPrep (Schrödinger, <http://www.schrodinger.com/>).

Docking

In the preliminary analysis step, several docking software and scoring functions have been tested (re-docking and cross-docking) for their ability to reproduce the protein–ligand complexes from the evaluation dataset: Gold [36] with the GoldScore, ChemScore, ChemPLP and ASP scoring functions, Vina [39] and AutoDock [37]. Default parameters were used in all cases for docking, except with Gold, where a search efficiency of 200% was used in order to better explore the conformational space. The binding sites were considered with Gold as spheres with a 20 Å radius around the C α atom of Ala288 (numbering from the 1OSV structure). With Vina and AutoDock, the binding sites were defined as a 40 $\times$ 40 $\times$ 40 Å³ cube centered on the same atom. The protein was considered to be either rigid, or with a few key residues (Leu291/Asn297/Met332/Arg335/Ser336/His451/Trp458 or Arg335 only) from the binding site as flexible. As a result of preliminary analysis, Gold with the ASP scoring function and the rigid protein was used in the Phase 1 predictions. In Phase 2, the rescoring of the FXR complexes was carried out using Gold with the ASP scoring function. For submission, the protein structures were converted into PDB format using UCSF Chimera [38], and the docking poses were converted into MOL format using CORINA (the MOL format corresponds to the SDF output format in CORINA). Unfortunately, the ligand conversion with CORINA was carried out initially without the option “-d no3d”, which led to the generation of new coordinates and therefore invalid conformations in our “rjyhz” submission. The results reported here (named “rjyhz_revised”) represent the correct poses, obtained with the option “-d no3d”.

Free energy calculations

The protein used for free energy calculation was taken from PDB database with PDB entry corresponding to 3FLI. Three-dimensional coordinates of ligands were built using UCSF Chimera [38], by superimposing common backbone on released FXR_17, FXR_10 and FXR_12 structures. In the set2, the structure solved by X-ray crystallography for FXR_12 had two alternative positions for the aromatic ring substituent (AA and AB). Both of them were considered in our calculations, and the one leading to the most favorable energy chosen for the submission. Alchemical free energies were calculated using Gromacs [17] and OPLS-AA force field [40, 41], some scripts from the PMX software [42–44] and some in house developed scripts. The main steps of this

Fig. 2 Chemical structures of the 36 FXR ligands included in Phase 1 for pose prediction (compound **FXR_33** was ultimately retired from the pose prediction analysis)

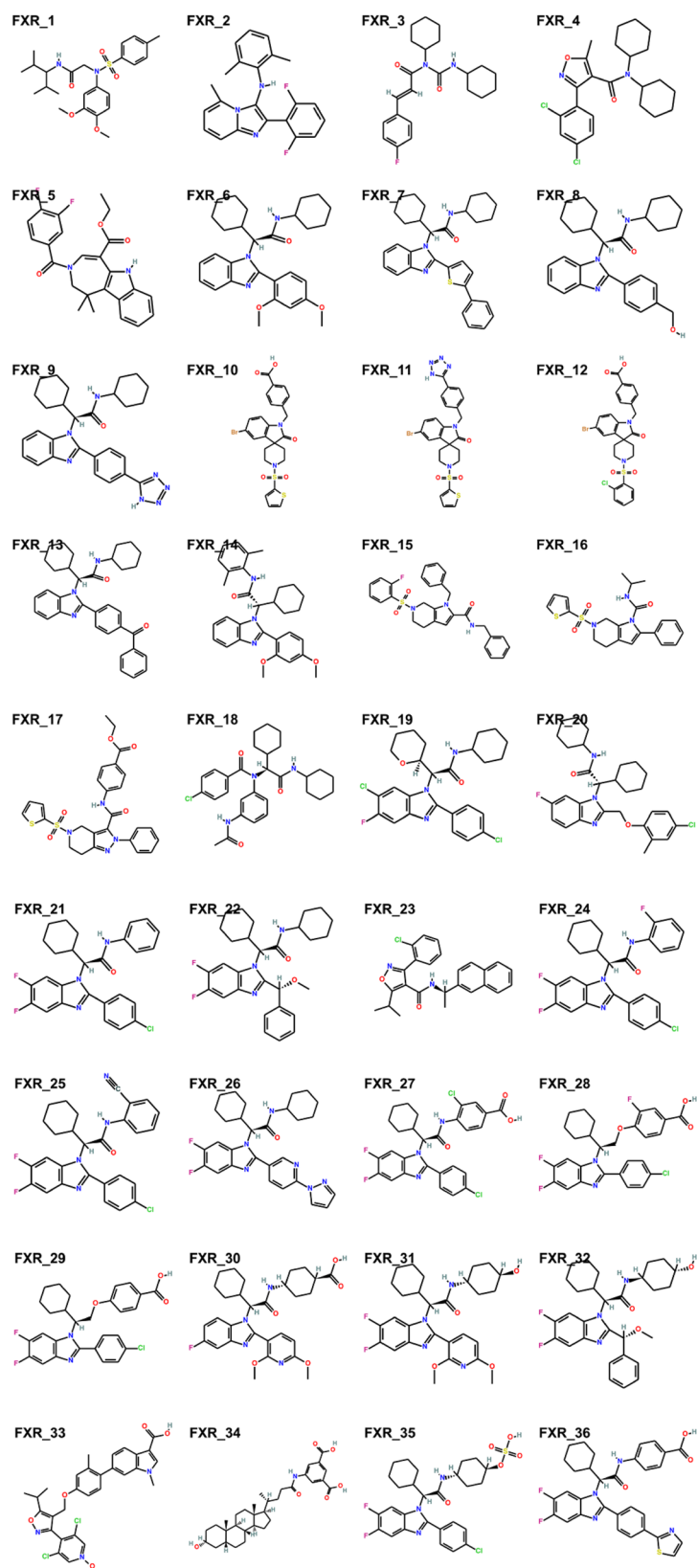
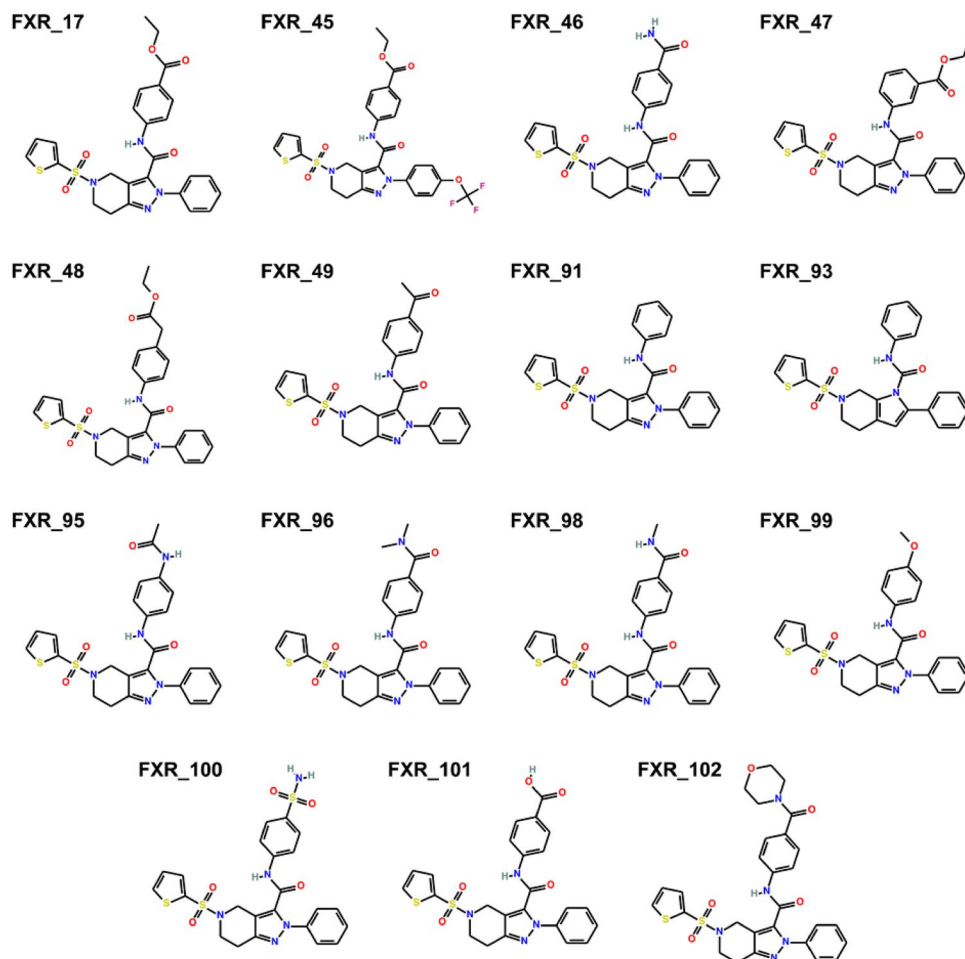


Fig. 3 Chemical structures of the 15 FXR ligands included in free energy set1 (sulfonamides)



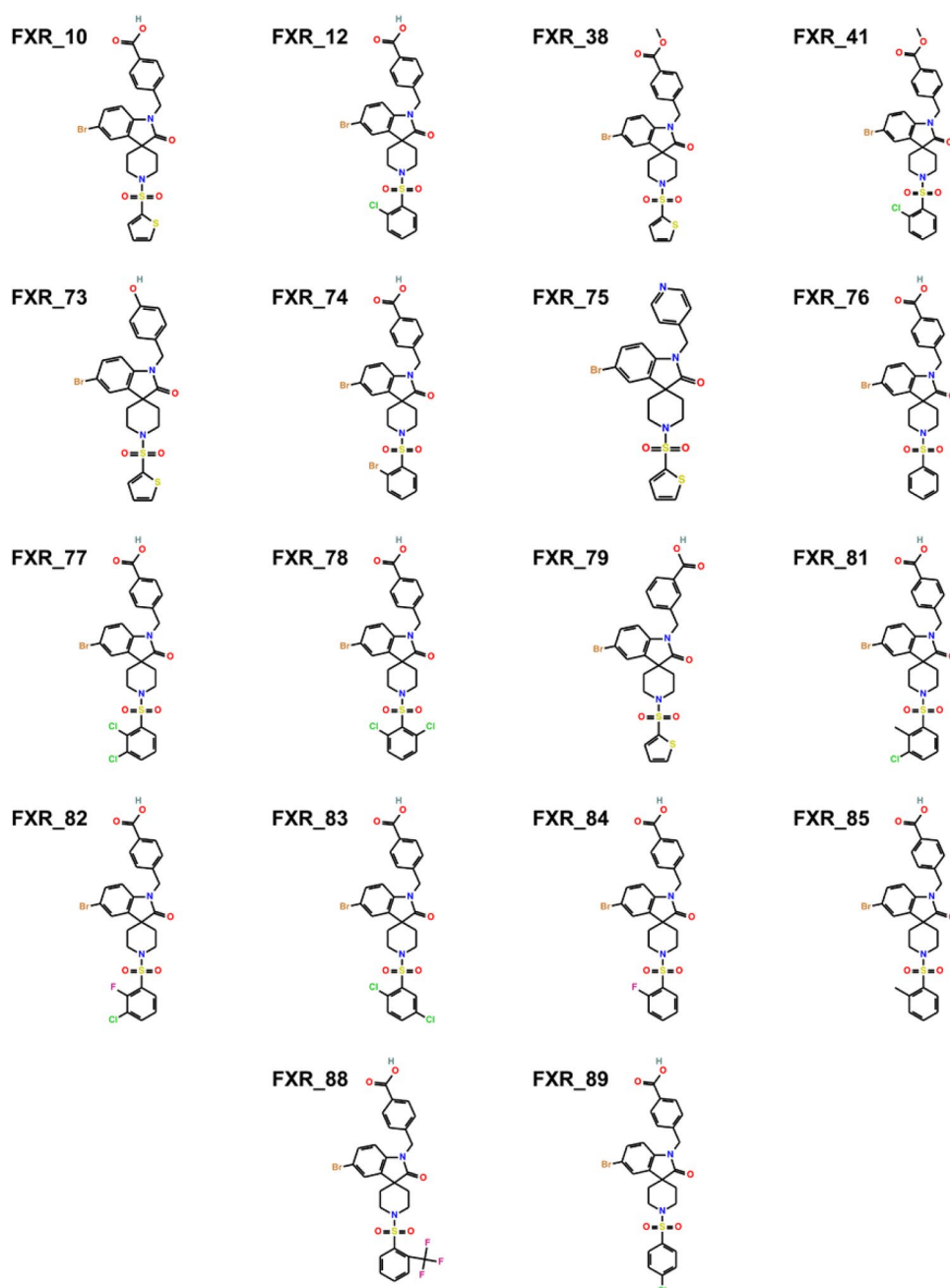
protocol are presented in Fig. 5. Hybrid structures and topologies were built using a modified version of the MOL2FF package developed in our team. Hybrid topologies represent simultaneously both ligands, the contribution of each structure being controlled by a parameter λ . For example, a λ value of 0 represents exclusively the ligand A, a λ value of 1 represents exclusively the ligand B, and a λ value of 0.3 represents a contribution of 70% of the ligand A and a contribution of 30% of the ligand B. FXR_91 was used as reference structure for set1, and FXR_10 or FXR_12 for set2. Equilibrium 10 ns MD simulations were performed for the two states (corresponding to λ 0 and 1), using Gromacs [17] and OPLS-AA force field [40, 41]. Snapshots from the equilibrium runs were extracted to spawn 100 simulations of 50 ps each to achemically morph between the two states of the system. The work values over every non-equilibrium transition were extracted and further used to estimate the free energy differences relying on the Crooks Fluctuation Theorem and utilizing Crooks-Gaussian Intersection as estimator. When the charge was the same in the two ligands considered for the achemical transformation, separate calculations were carried out for the transformation A into B

of the protein–ligand complex and of the ligand alone, the relative free energy of binding being the difference between the corresponding work for these two transformations (see Figures S2 and S3 in the Electronic Supplementary Material file). When the charge was different between the two ligands, a single box containing the protein–ligand complex and the ligand alone, separated by 30 Å, was considered. The ligand from the complex was converted from state A into B, whereas the ligand alone was converted simultaneously from state B into A. In these conditions, the overall charge of the system was conserved during the whole simulation, and the relative free energy of binding between ligands corresponds to the global work for this system (see Figure S4 in the Electronic Supplementary Material file).

Graphics

Chemical structures were depicted using CACTVS Chemo-informatics Toolkit v3.409 (Xemistry, <http://www.xemistry.com/>), images for protein structures were generated using PyMol 1.8.1 (Schrödinger, <http://www.pymol.org/>)

Fig. 4 Chemical structures of the 18 FXR ligands included in free energy set2 (spiro compounds)



and histograms were obtained using the R package (<http://www.r-project.org>).

Statistics

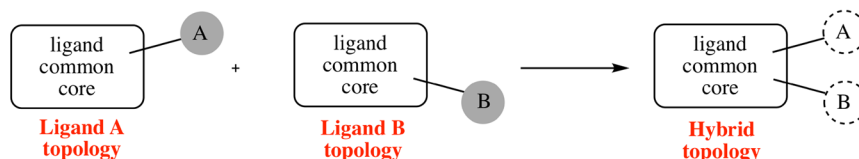
Statistics were computed using the online tools available at <http://www.sthda.com/english/rsthda/correlation.php>.

Chemoinformatics

Tanimoto similarities were computed using CACTVS Chemoinformatics Toolkit v3.409 (Xemistry, <http://www.xemistry.com/>).

Fig. 5 The main steps of the protocol used for the calculation of alchemical free energies

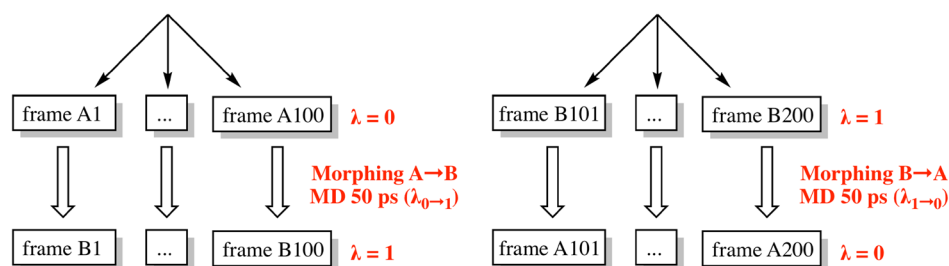
Step 1: Generation of hybrid topology



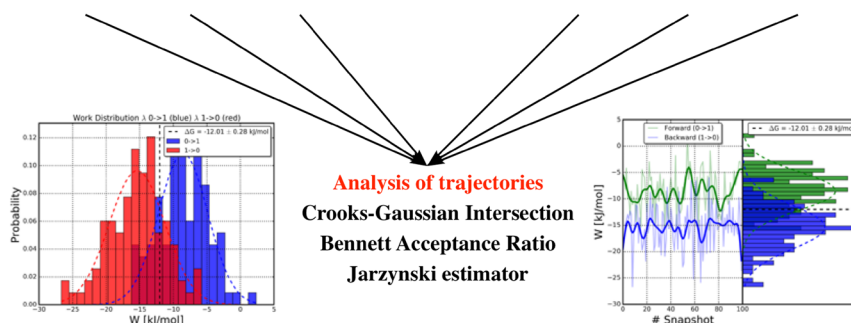
Step 2: Conformational sampling using equilibrium MD



Step 3: Structure morphing using non-equilibrium MD



Step 4: Free energy estimation



Results and discussion

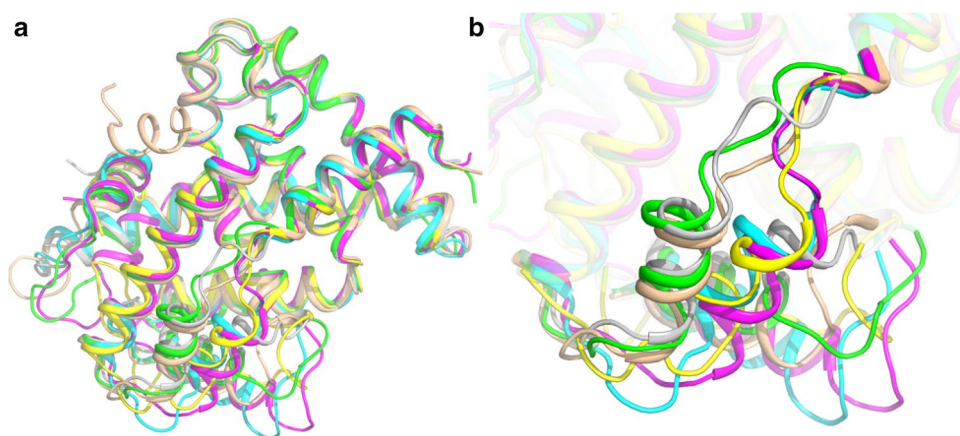
In our previous participations to the SAMPL3 (2011) [45], SAMPL4 (2013) [46], CSAR (2014) [47] and D3R Grand Challenge (2015) [48] docking and virtual screening challenges we followed an approach involving two steps. The first step consists in a preliminary analysis of information available in literature (structural, and in some cases enzymatic data), which allows the identification of the best combination of docking software and scoring function that are suited for studying the system of interest. In the second step, the combination of docking software and scoring function is used to predict the binding modes (pose prediction) and the relative affinities of ligands (scoring). As

our previous studies [45–48] highlighted the importance of using enhanced genetic algorithm parameters for docking (a search efficiency of 200%), in this work we used the same parameters in order to ensure an adequate conformational sampling of docking conformations.

Preliminary analysis

We found 27 crystal structures of FXR that were available in the PDB [34]. These structures were organized in five distinct groups, according to their conformation and the ligand present in the binding site (see the Electronic Supplementary Material for a complete list of these structures and the exact composition of each group). A representative structure

Fig. 6 Representative 6 FXR PDB structures superimposed: **a** general view, showing a very good global conservation of structural features; **b** zoom on residues 258–285 and 335–358, highlighting the conformational flexibility of these fragments. The structures are represented as follows: 1OSV (*green*), 3FLI (*cyan*), 3OLF (*magenta*), 4WVD (*yellow*), FXR apo (*wheat*), 3HC5 (*gray*)



was selected from each group, based on the crystal structure resolution and the lack of missing residues. The three-dimensional structure of protein in these structures is well conserved, with the exception of two fragments (residues 258–285 and 335–358) that are very flexible (Fig. 6). These five structures, together with the apo FXR structure provided by the organizers, constitute our evaluation dataset, which was used in re-docking and cross-docking calculations using the FXR ligands from all the 27 structures available and several combinations of docking software and scoring functions: Gold with the GoldScore, ChemScore, ChemPLP and ASP scoring functions, Vina and Autodock.

RMSD values compared with the native ligands from the crystallographic structures were calculated for all docking poses. In order to evaluate the accuracy of docking and scoring, we have considered the lowest RMSD value and the RMSD value of the best ranking pose for each combination protein–ligand-(docking software)-(scoring function). AutoDock provided very poor results, with most of the docking conformations positioned outside the binding site, whereas Gold/ASP, followed by Gold/GoldScore and Vina, could reproduce rather well the native protein–ligand complexes, especially in the cross-docking calculations. As expected, the re-docking results outperformed the cross-docking results. It was also observed that the combination of a protein and a ligand belonging to the same group was more favorable than a combination of a protein and a ligand from different groups.

Pose prediction and scoring

The 102 FXR ligands from the D3R Grand Challenge 2 dataset containing 180 ligands were docked on the 6 representative FXR structures shown in Fig. 6 using Gold with the ASP scoring function. For each ligand, the best-ranked docking conformation was selected and the overall ranking was submitted, as well as the coordinates for the ligands FXR_1 to FXR_36. For the ligands belonging to a group

for which crystal structures were available (e.g. benzimidazoles, isoxazoles), the RMSD was calculated using the largest common fragment, and the conformations with the best RMSD were selected for a second submission. The RMSD calculation was realized using an in house developed script based on CACTVS Chemoinformatics Toolkit. The poses from the spiro and sulfonamides groups were visually inspected using UCSF Chimera. Only the 3FLI and the APO structures provided docking poses with a carboxylate group (that is present in most spiro structures and in FXR_101 from the sulfonamides group) interacting with Arg335. This was considered as the correct orientation, since most of the crystalized ligands show the same kind of interaction. Overall, poses obtained with the structure 3OLF were selected for benzimidazoles, with 1OSV for steroids, with 3HC5 for isoxazoles and with 3FLI for all others.

The performance of submissions for pose prediction (best RMSD and RMSD of pose 1) is presented in Fig. 7, showing a relatively good result that we obtained in this category compared with the other participants.

Our scoring results for the two submissions in Phase 1 were very modest, with Kendall Tau values of 0.13 and 0.072. Table S1 from the Supplementary Information file shows the rank of the best RMSD pose for compounds with existing reference structural data (53 compounds out of 102 compounds from the dataset). A mean value of 4.68 (out of 10 poses in each case) was obtained for this rank, which is quite low. If we also consider that for the remaining 49 compounds with no reference structure available we have no information about the docking reliability, these data altogether might explain the incorrect scoring prediction.

The crystallographic structures of the 36 FXR complexes proposed for pose prediction were released at the end of Phase 1. A comparison of several representative docking poses and the corresponding crystallographic conformations is provided in Fig. 8. We predicted well the conformation of most benzimidazoles, but the other three groups (isoxazoles, spiro compounds and sulfonamides) were more challenging,

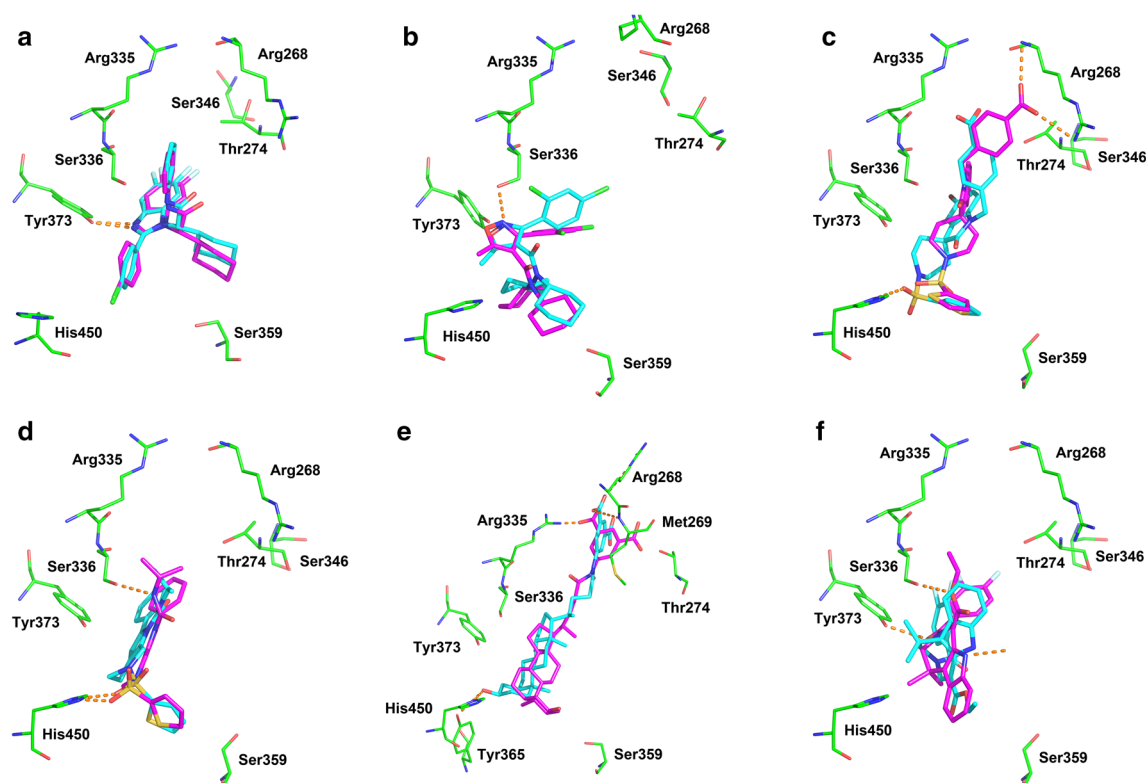


Fig. 8 Comparison of our docking poses (cyan) with crystal structure conformations (*magenta*) for representative FXR ligands from different families: **a** benzimidazoles (FXR_21/3OLF, RMSD 0.97 Å); **b** isoxazoles (FXR_4/3HC5, RMSD 3.87 Å); **c** spiro compounds

(FXR_10/3FLI, RMSD 2.85 Å); **d** sulfonamides (FXR_16/3FLI, RMSD 2.03 Å); **e** miscellaneous (FXR_34/1OSV, RMSD 3.76 Å); **f** miscellaneous (FXR_5/3FLI, RMSD 4.70 Å)

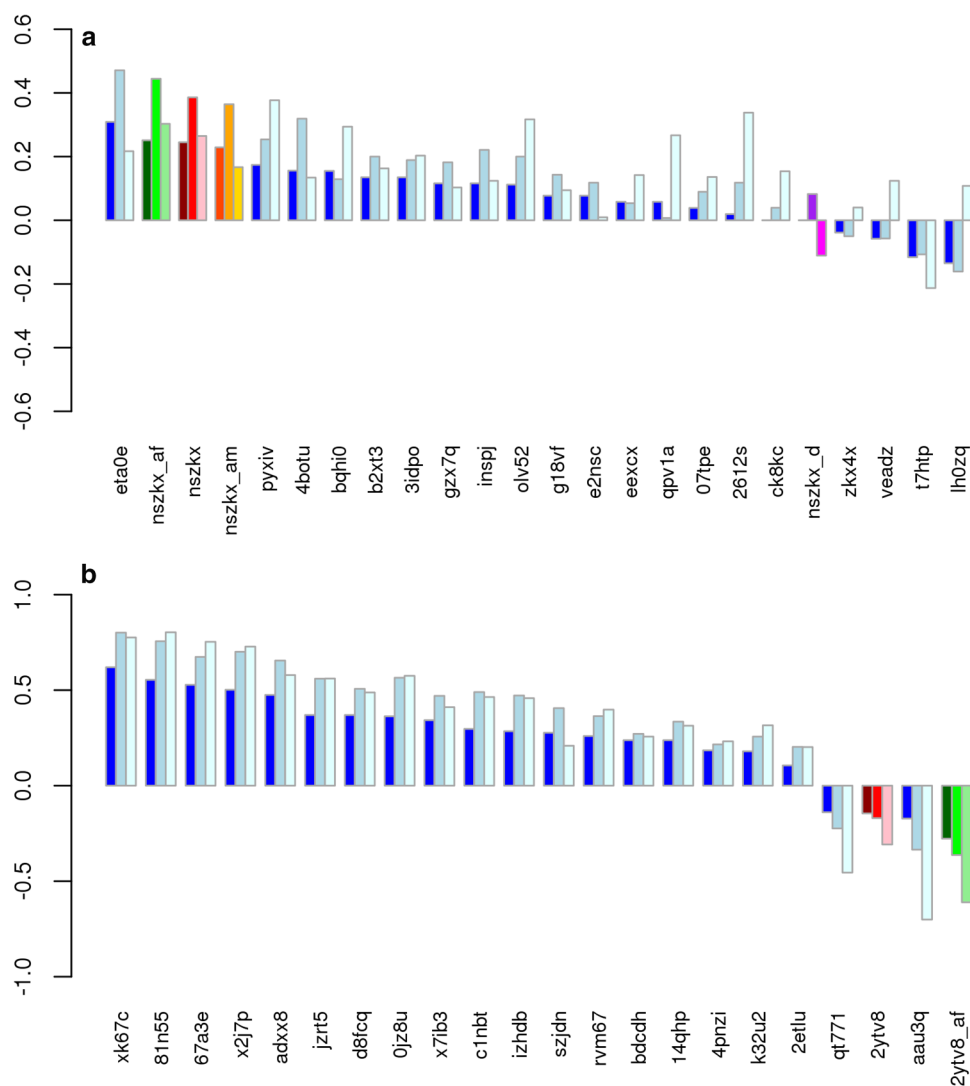
initial coordinates (which represents Phase 1 calculations carried out retrospectively), no correlation was obtained. This corroborates with the pose prediction results, showing that no good quality predictions can be obtained from inaccurate docking poses.

For set2, the results were quite disappointing, with negative correlations with OPLS-AA before and after the correction. Apparent better correlations were obtained with OPLS-AA using the docking poses and with AMBER/GAFF, but they are not representative since they were computed only for 12 and 6 values, all of them belonging to the FXR_12 subset (see Table 2 for the compounds belonging to the FXR_10 and FXR_12 subsets), so we decided not to represent them in Fig. 9.

We tried to find a rational explanation for the discrepancy of the results obtained for set1 and set2, using the same protocol. Among the possible hypotheses, we can mention: (i) the intrinsic greater structural diversity in set2 compared with set1; (ii) incorrect force field parameters and (iii) insufficient conformation sampling of ligands.

To validate the first hypothesis, we computed the Tanimoto similarity matrix for set1 and set2 (see Tables S2 and S3 in Electronic Supplementary Material). The global mean values of Tanimoto similarity for the two datasets are very close, 0.88 and 0.86, respectively, suggesting a similar degree of diversity. However, a visual inspection of the two datasets shows that set1 is quite homogeneous, with variations on the substitution pattern of a single phenyl ring. On the other hand, compounds from set2 contain variations on two fragments: one can be a diversely substituted phenyl ring, and the other can be either a thienyl ring or a diversely substituted phenyl ring. According to the presence or not of the thienyl ring, set2 can be divided into two subsets, which have FXR_10 and FXR_12 as representative compounds. We computed the statistics separately on these two subsets and the results are presented in Table 3. Compared with the whole set2, only a small improvement in the correlation with experimental data is observed for the FXR_12 subset. However, for the FXR_10 subset we observe almost a perfect anticorrelation with the experimental data. Overall, this

Fig. 9 Performance of Phase 2 free energy submissions for set1 (a) and set2 (b). The correlation coefficients are represented as follows: Kendall tau in blue, Spearman rho in light blue and Pearson r in cyan. Our submissions are colored in dark red, red and pink, respectively (nszkkx and 2ytv8). The results obtained on recomputed simulations after fixing a bug in the hybrid topologies are represented in different shades of green (nszkkx_af and 2ytv8_af). The results from simulations using docking poses instead of crystal structures (equivalent of Phase 1 calculations carried out retrospectively) are represented in magenta (nszkkx_d). The results from simulations using AMBER/GAFF force field instead of OPLS-AA are represented in orange (nszkkx_am)



analysis shows that the differences between the structural diversity of set1 and set2 are too small to be discriminated by descriptors such as Tanimoto similarity, but the set2 is more diverse and can be divided in two subsets. One of these subsets, containing a thienyl substituent, has a major negative impact in the prediction of free energies for set2.

To evaluate the pertinence of the second hypothesis, we analyzed the conformational distribution of the ligands FXR_17, FXR_10 and FXR_12, as representative structures for set1 and the two subsets of set2, in two force fields, OPLS-AA and AMBER/GAFF. In each case, we extracted and superimposed all the 501 conformations from the 10 ns molecular dynamics simulation of the ligand alone in water. The result is presented in Figure S5 (Electronic Supplementary Material).

For compound FXR_17, we observe 4 main differences between the distributions OPLS-AA (a) and AMBER/

GAFF (b): (i) the phenyl ring is mostly parallel with the bicyclic system in a, and perpendicular in b; (ii) the amide group is mostly perpendicular with the bicyclic system in a, and parallel in b; (iii) the distribution of the thienyl ring around the dihedral C–N–S–C is restricted to a very narrow window in a, whereas in b there are two larger windows in opposite positions, showing in the latter case an unrestricted exchange between these two positions; (iv) in a the thienyl ring shows equivalent populations of both faces, whereas in b the rotation around the dihedral N–S–C–S is very much restricted. However, as the predictions of set1 using either OPLS-AA or AMBER/GAFF are very similar (see Fig. 9a; Table 1), these differences should not have a major contribution or, more probably, should cancel mutually.

For compound FXR_10, we observe in a a restricted rotation around the C–N–S–C dihedral and, in

Table 1 Free energies computed for set1

	Experimental IC50 (μ M)	OPLS-AA before correction (nszkkx)		OPLS-AA after correction (nszkkx_af)		OPLS-AA with docking poses (nszkkx_d)		AMBER/GAFF (nszkkx_am)	
		$\Delta\Delta G$ (kJ/mol)	$\Delta\Delta G$ error (kJ/mol)	$\Delta\Delta G$ (kJ/mol)	$\Delta\Delta G$ error (kJ/mol)	$\Delta\Delta G$ (kJ/mol)	$\Delta\Delta G$ error (kJ/mol)	$\Delta\Delta G$ (kJ/mol)	$\Delta\Delta G$ error (kJ/mol)
FXR_17	0.79	-9.32	1.16	-18.35	1.12	-17.02	1.43	-10.30	1.92
FXR_45	28.85	-3.14	2.33	-21.21	2.15	-38.07	1.57	-30.63	2.33
FXR_46	62.37	-3.81	21.82	-12.98	1.26	-14.93	21.24	-4.09	1.82
FXR_47	20.96	NA	NA	-18.61	2.41	19.33	1.85	-4.02	3.04
FXR_48	100.00	NA	NA	5.36	1.75	-9.26	1.89	NA	NA
FXR_49	100.00	-3.73	1.48	-9.47	1.02	-18.39	0.85	-1.95	1.38
FXR_91	29.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
FXR_93	46.66	-8.98	1.26	-4.04	0.39	-5.29	0.46	2.91	0.55
FXR_95	32.17	-8.39	1.45	-6.63	1.21	-11.51	1.61	-11.16	1.45
FXR_96	58.86	-19.12	1.62	-21.36	2.15	-16.79	1.86	NA	NA
FXR_98	13.14	-21.07	1.42	-12.95	0.97	-22.69	1.49	-22.68	2.05
FXR_99	100.00	-6.34	1.59	-11.07	0.56	-12.02	2.03	-9.77	1.17
FXR_100	19.14	-15.86	2.49	-25.81	3.05	-13.11	3.60	-4.52	2.90
FXR_101	27.64	-36.24	3.38	-27.94	3.69	-9.78	3.46	NA	NA
FXR_102	29.23	-0.15	2.85	13.45	2.17	17.31	2.97	15.34	2.97
		Correlation coefficient	p value	Correlation coefficient	p value	Correlation coefficient	p value	Correlation coefficient	p value
Kendall's rank correlation tau		0.2452	0.2455	0.2512	0.1962	0.0000	1.0000	0.2290	0.3025
Spearman's rank correlation rho		0.3851	0.1937	0.4444	0.0970	0.0824	0.7702	0.3643	0.2444
Pearson's product-moment correlation r		0.2648	0.3819	0.3029	0.2726	-0.1110	0.6936	0.1668	0.6044

FXR_91 was used as reference compound

opposition with FXR_17, an impossible rotation around the N-S-C-S dihedral, probably because of the close proximity of the amide oxygen. In the case of **b**, we observe a free rotation around the C-N-S-C dihedral and a restricted rotation around the N-S-C-S dihedral, similar with FXR_17.

Finally, we observe for FXR_12 a restricted rotation around the C-N-S-CA dihedral in **a** and a free rotation around the N-S-CA-CA dihedral (the chlorine substituent is positioned equally on both sides), whereas in **b** the rotation around the C-N-S-CA dihedral is relatively free in the conditions of simulation, but the rotation around the N-S-CA-CA dihedral is almost completely restricted.

These results suggest the possible existence of two non-optimal dihedrals associated with the sulfonamide group, similarly with a recent report regarding the incorrect conformational sampling of linezolid [49]. For set1, their influence might be compensated by two other dihedrals, which is not the case for set2. Additionally, in the FXR_12 subset there are two atropisomers that can contribute to the overall

binding energy, whereas in our calculations we have considered only one, the most favorable.

The third hypothesis, insufficient conformation sampling of ligands, is not very probable given the length of our molecular dynamics simulations and the relative rigidity of the ligands. If the conformation space is not sampled correctly, this should be more due to inadequate force field parameters than to insufficient length of simulations. Along the same lines, in a few specific cases, the standard deviation of our predictions is unusually high (see the " $\Delta\Delta G$ error" columns in Tables 1, 2), especially for set2.

Conclusions

We used in this work a protocol in two steps, involving an initial analysis of the available structural data from the PDB, which allows the selection of the most appropriate combination of docking software and scoring function. Subsequent docking calculations showed that the pose

Table 2 Free energies computed for set2

	Experimental IC50 (μM)	OPLS-AA before correc- tion (2ytv8)		OPLS-AA after correction (2ytv8_af)		OPLS-AA with docking poses		AMBER/GAFF		
		ΔΔG (kJ/mol)	ΔΔG error (kJ/mol)	ΔΔG (kJ/mol)	ΔΔG error (kJ/mol)	ΔΔG (kJ/mol)	ΔΔG error (kJ/mol)	ΔΔG (kJ/mol)	ΔΔG error (kJ/ mol)	
FXR_10*	5.64	−10.69	0.81	−4.85	0.48	NA	NA	NA	NA	
FXR_12	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
FXR_38*	100.00	−27.49	−	−18.86	−	NA	NA	NA	NA	
FXR_41	100.00	−15.19	4.15	−3.24	4.27	7.55	4.73	NA	NA	
FXR_73*	11.22	10.77	−	−14.83	−	NA	NA	NA	NA	
FXR_74	0.66	−3.16	−	−2.57	0.16	NA	NA	NA	NA	
FXR_75*	100.00	−71.30	−	−32.41	−	NA	NA	NA	NA	
FXR_76	41.83	−0.01	0.62	−1.78	66.29	2.78	0.22	12.07	0.55	
FXR_77	0.25	−2.35	0.35	−2.47	0.23	−1.54	2.89	−6.09	0.67	
FXR_78	0.03	−15.94	1.08	−5.89	19.75	1.07	105.65	NA	NA	
FXR_79*	4.15	2.39	−	14.84	−	NA	NA	NA	NA	
FXR_81	2.69	−11.09	0.73	−10.97	18.40	−11.58	65.68	NA	NA	
FXR_82	0.18	7.14	0.85	2.60	0.58	4.83	0.52	2.69	1.99	
FXR_83	0.33	−1.75	0.31	5.02	0.77	3.75	6.78	NA	NA	
FXR_84	4.54	−5.87	0.66	4.09	17.88	3.23	10.41	NA	NA	
FXR_85	0.30	−9.67	0.40	−5.10	90.68	−1.96	0.29	−0.41	1.60	
FXR_88	0.54	−3.73	0.40	0.81	0.33	2.84	0.36	NA	NA	
FXR_89	0.74	−10.31	75.22	−4.15	75.34	−2.27	40.07	4.80	0.87	
			Correlation coefficient	p value	Correlation coefficient	p value	Correlation coefficient	p value	Correlation coefficient	p value
Kendall's rank correlation tau			−0.2772	0.1107	−0.2772	0.1107	0.0303	0.9466	0.4667	0.2722
Spearman's rank correlation rho			−0.3320	0.1784	−0.3630	0.1387	0.1748	0.5883	0.5429	0.2972
Pearson's product-moment correlation r			−0.6900	0.0015	−0.6105	0.0071	0.4651	0.1276	0.8012	0.0554

The FXR_10 subset contains the five compounds marked with a star, and the FXR_12 subset contains the remaining compounds. FXR_10 and FXR_12 were used as reference compounds for each subset, then all free energies from subset FXR_10 were translated relative to FXR_12

Table 3 Statistics computed for the subsets FXR_10 and FXR_12 of set2

FXR_10 subset	OPLS-AA before correction		OPLS-AA after correction	
	Correlation coefficient	p value	Correlation coefficient	p value
Kendall's rank correlation tau	-0.5270	0.2065	-0.9487	0.0230
Spearman's rank correlation rho	-0.6669	0.2189	-0.9747	0.0048
Pearson's product-moment correlation r	-0.8377	0.0766	-0.7737	0.1247
FXR_12 subset	Correlation coefficient		Correlation coefficient	
	Correlation coefficient	p value	Correlation coefficient	p value
Kendall's rank correlation tau	-0.2564	0.2519	-0.0513	0.8577
Spearman's rank correlation rho	-0.2692	0.3733	-0.0330	0.9206
Pearson's product-moment correlation r	-0.3261	0.2769	-0.0902	0.7696

See Table 2 and text for the list of compounds included in each subset

prediction can be carried out with a certain precision, but this is dependent on the specific nature of the ligands. The correct ranking of docking poses is still a problem and cannot be successful in the absence of good pose

predictions. Our free energy calculations on two different subsets provided contrasted results, which might have the origin in non-optimal force field parameters associated with the sulfonamide chemical moiety.

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Electronic Supplementary Material

Blinded evaluation of farnesoid X receptor (FXR) ligands binding using molecular docking and free energy calculations

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Author Contributions

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Protein Data Bank (PDB) structures available

27 structures were available in the PDB for FXR at the moment when the D3R Grand Challenge 2 took place. They were organized in 5 distinct groups, according to the type of ligand and the binding site conformation. The representative structure for each group (based on the crystal structure resolution and lack of missing residues) is colored in red.

Group 1 (isoxazoles): 3dct, 3dcu, 3gd2, **3hc5**, 3hc6, 3p88, 3p89, 3rut, 3ruu, 3rvf

Group 2 (benzimidazoles): 3okh, 3oki, **3olf**, 3omk, 3omm, 3oof, 3ook

Group 3 (FXR_5-like): 3l1b, **3fli**

Group 4 (steroid, FXR_34-like): 3bej, **1osv**, 1ot7, 4qe6

Group 5 (miscellaneous): 1osh, 4oiv, 4qe8, **4wvd**

FXR ligands regroupment according to their chemical structure

Isoxazoles: FXR_4, FXR_23, FXR_33, FXR_65

Benzimidazoles: FXR_6, FXR_7, FXR_8, FXR_9, FXR_13, FXR_14, FXR_19, FXR_20, FXR_21, FXR_22, FXR_24, FXR_25, FXR_26, FXR_27, FXR_28, FXR_29, FXR_30, FXR_31, FXR_32, FXR_35, FXR_36, FXR_37, FXR_39, FXR_40, FXR_42, FXR_50, FXR_51, FXR_52, FXR_53, FXR_54, FXR_55, FXR_56, FXR_57, FXR_58, FXR_59, FXR_60, FXR_61, FXR_62, FXR_63, FXR_64, FXR_66, FXR_67, FXR_68, FXR_69, FXR_70, FXR_71, FXR_72

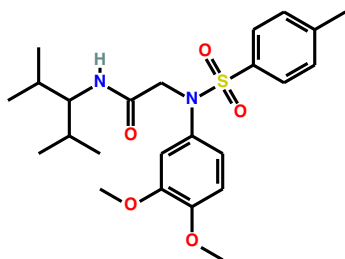
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Sulfonamides: FXR_15, FXR_16, FXR_17, FXR_43, FXR_44, FXR_45, FXR_46, FXR_47, FXR_48, FXR_49, FXR_90, FXR_91, FXR_92, FXR_93, FXR_94, FXR_95, FXR_96, FXR_97, FXR_98, FXR_99, FXR_100, FXR_101, FXR_102

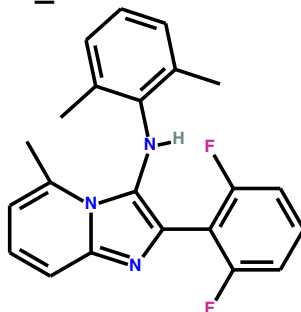
Miscellaneous: FXR_1, FXR_2, FXR_3, FXR_5, FXR_18, FXR_34

Figure S1. Chemical structures of the entire FXR dataset, containing 102 ligands used for ranking prediction.

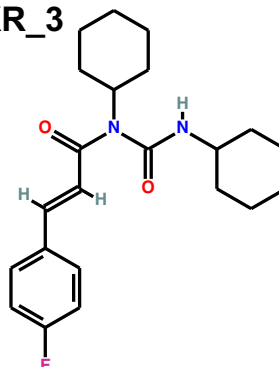
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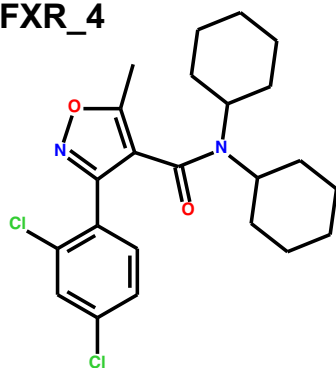
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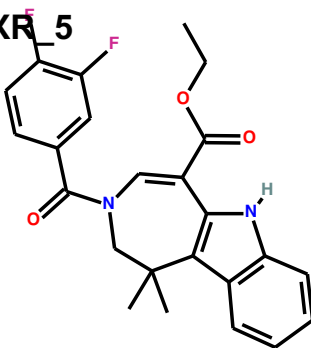
FXR_3



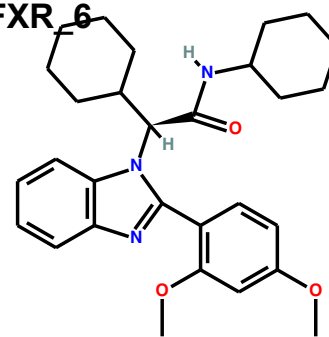
FXR_4



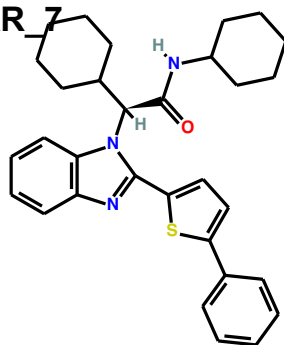
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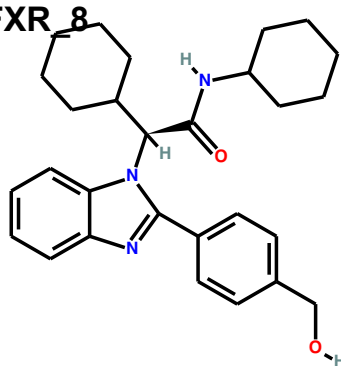
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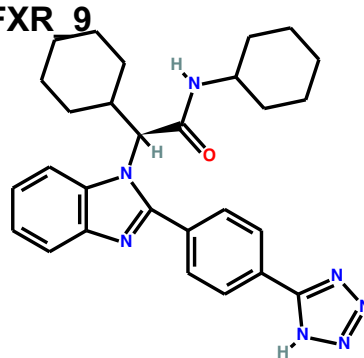
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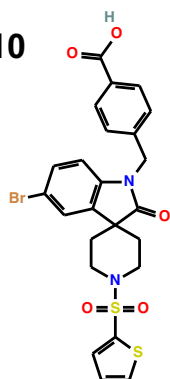
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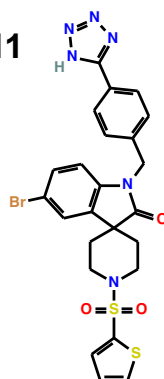
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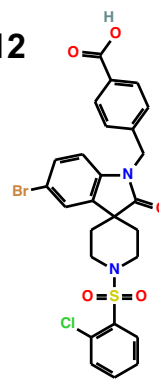
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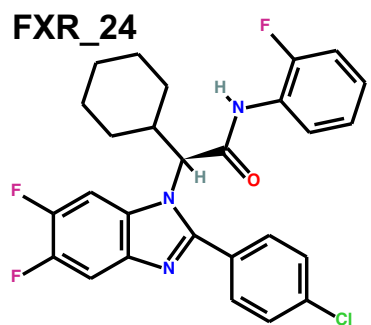
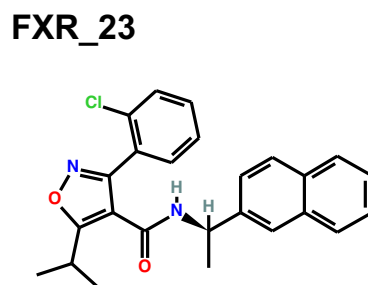
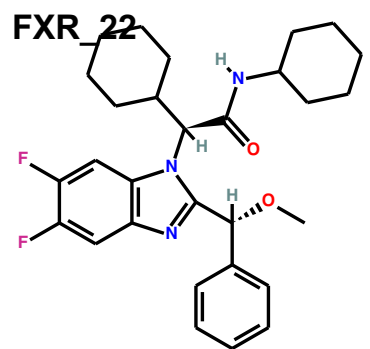
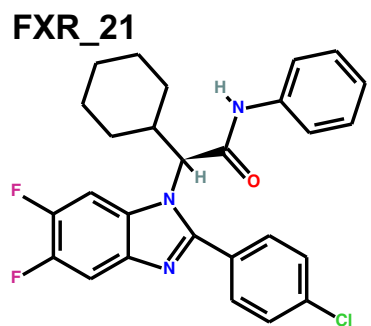
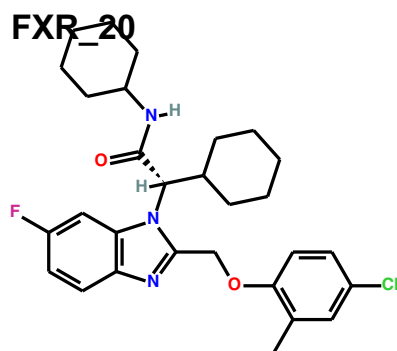
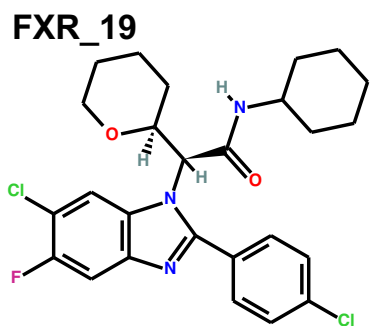
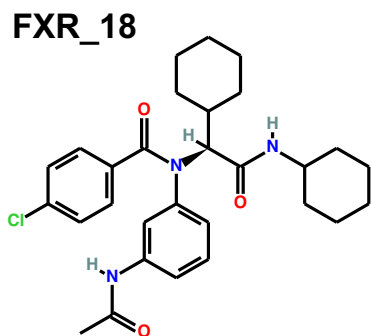
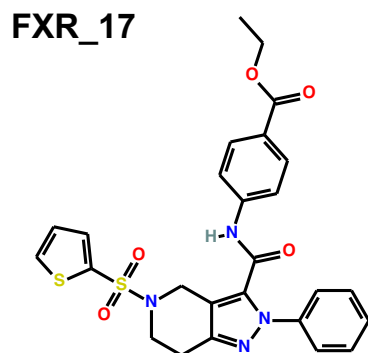
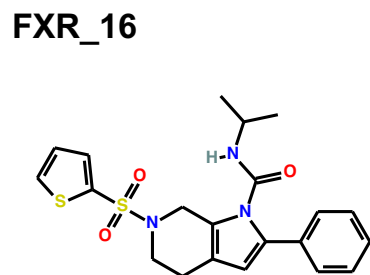
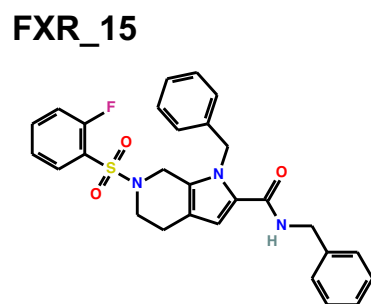
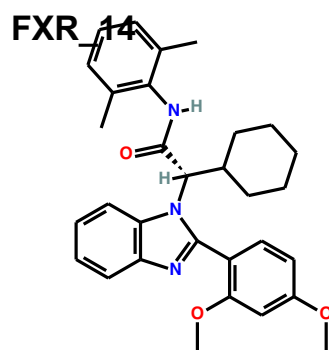
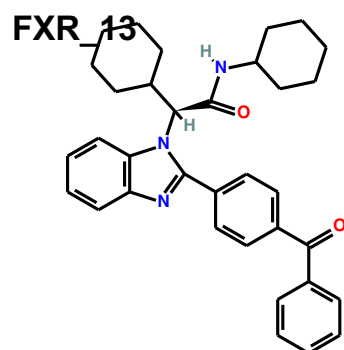


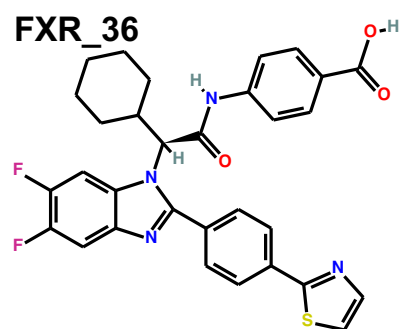
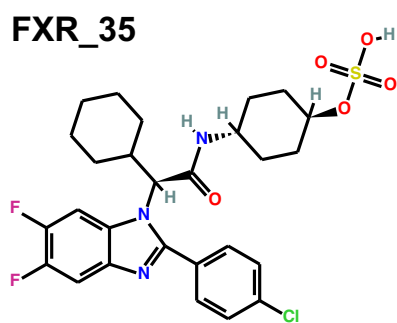
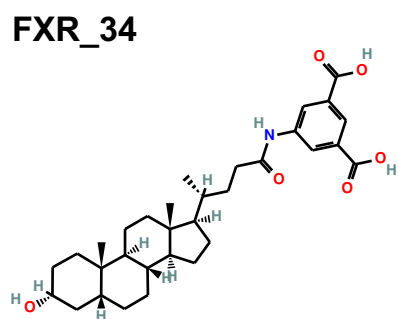
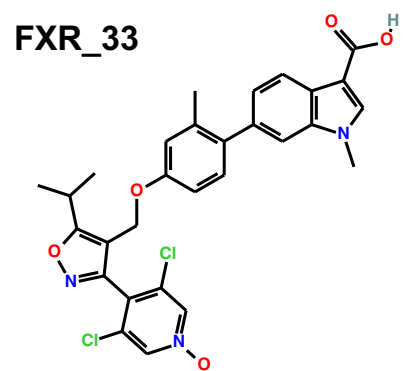
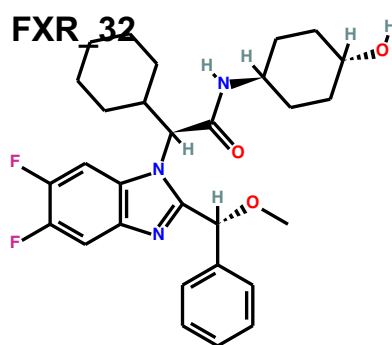
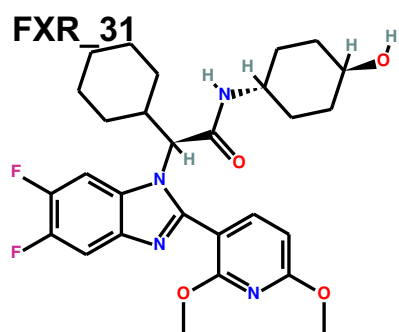
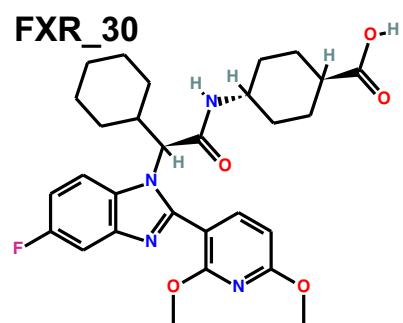
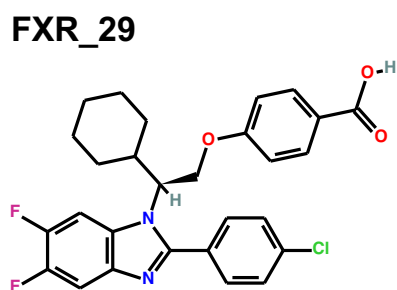
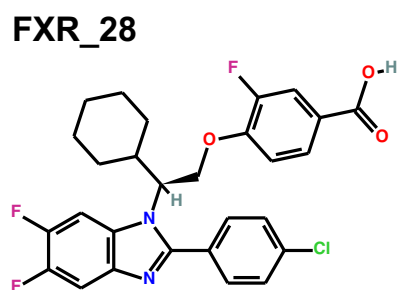
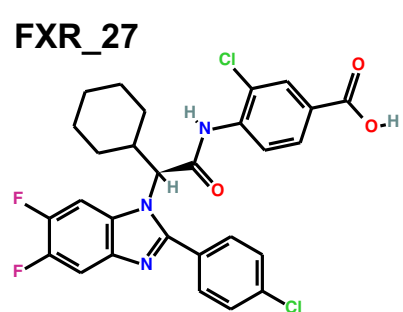
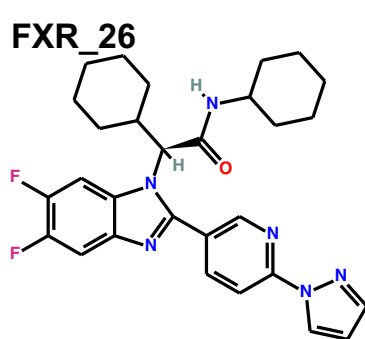
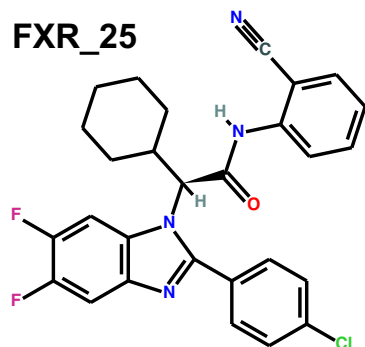
FXR_11

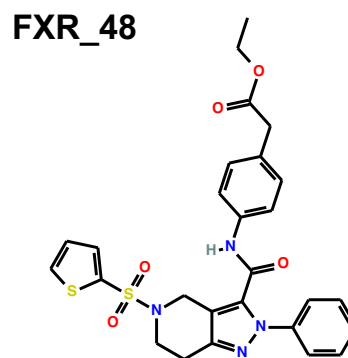
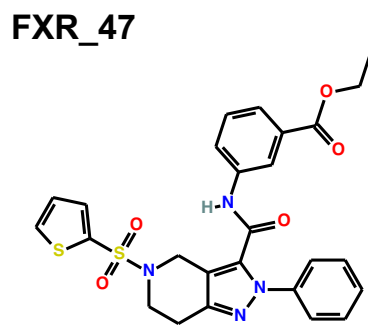
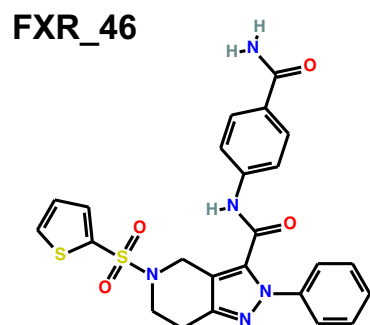
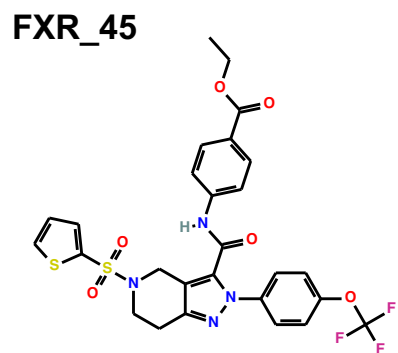
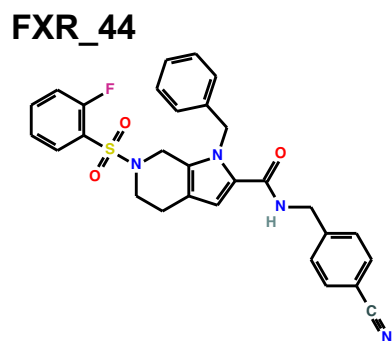
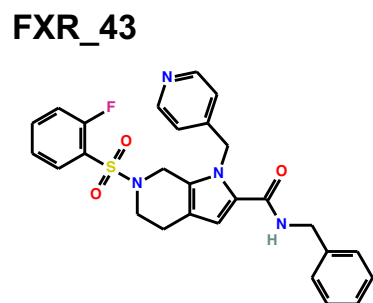
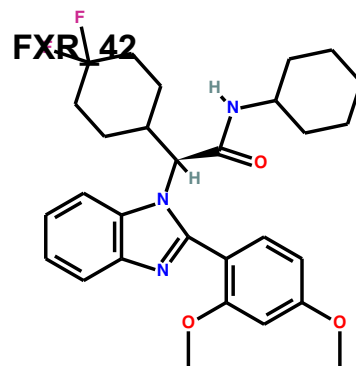
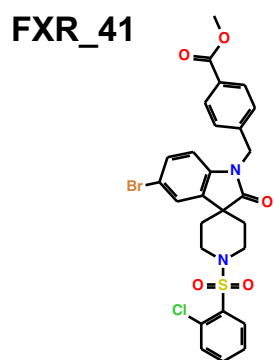
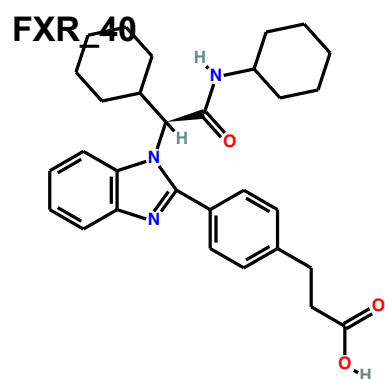
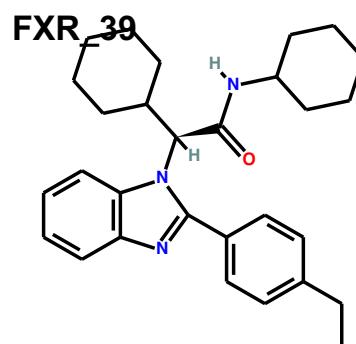
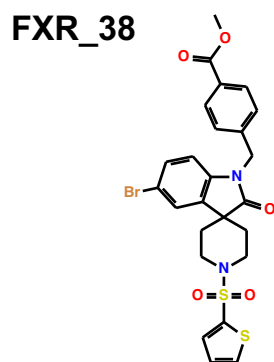
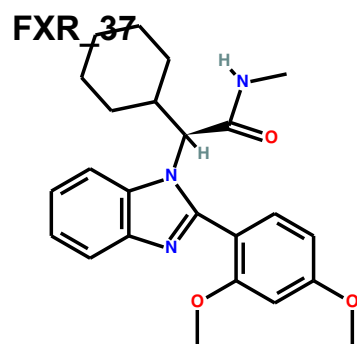


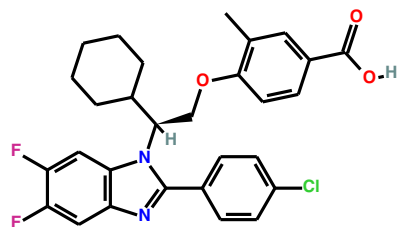
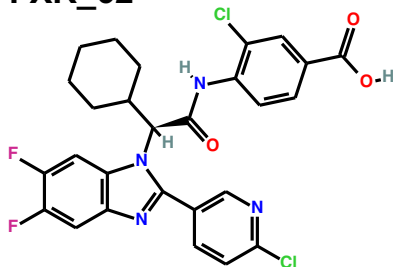
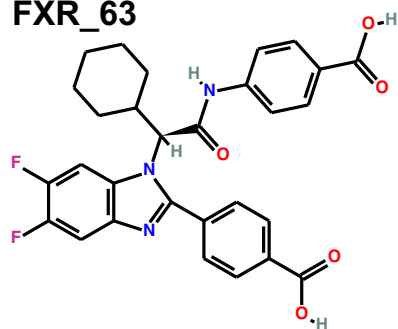
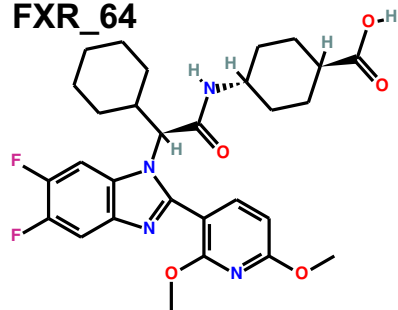
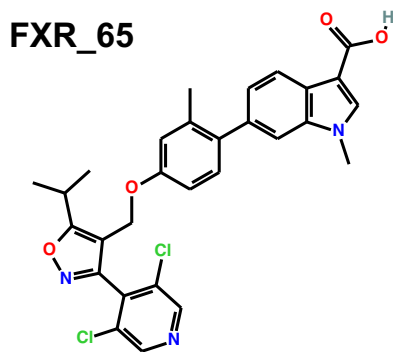
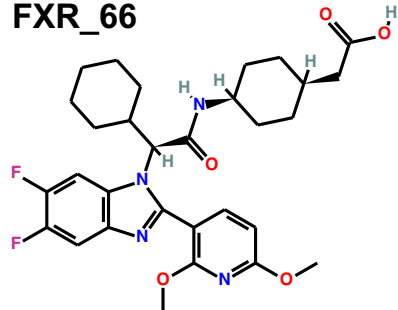
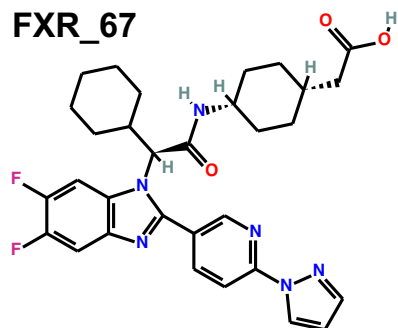
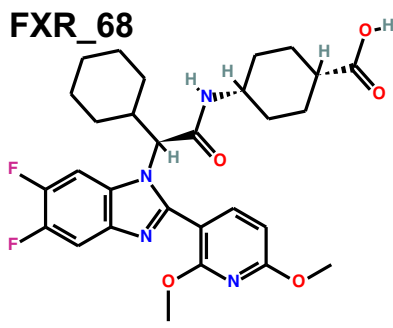
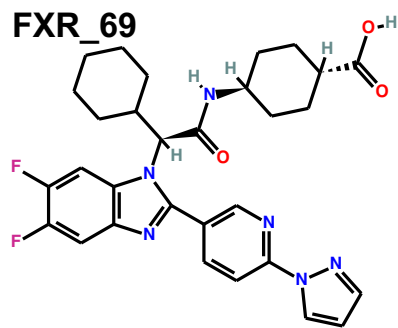
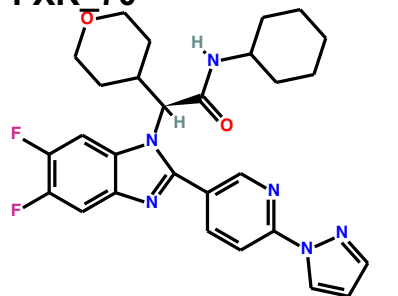
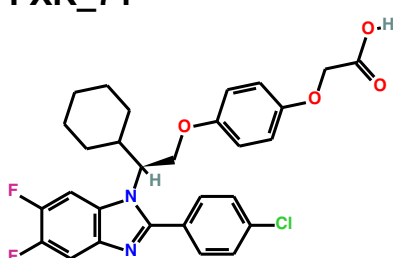
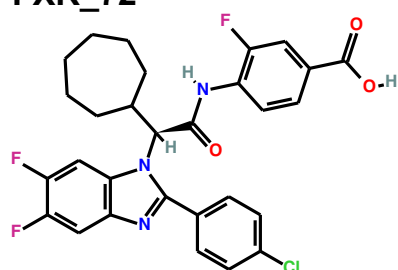
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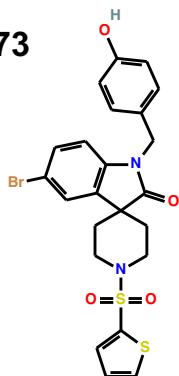




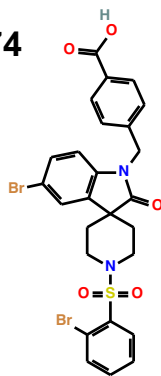


FXR_61**FXR_62****FXR_63****FXR_64****FXR_65****FXR_66****FXR_67****FXR_68****FXR_69****FXR_70****FXR_71****FXR_72**

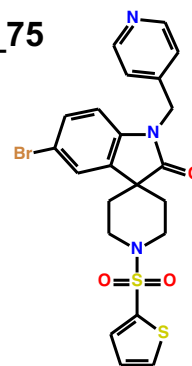
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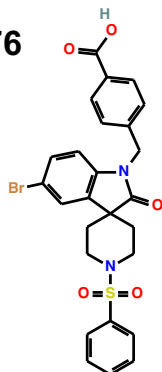
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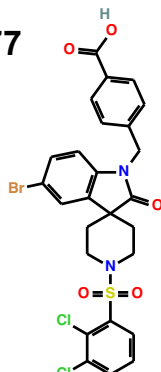
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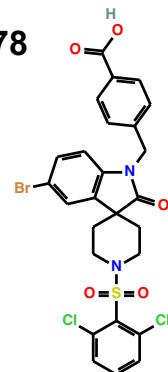
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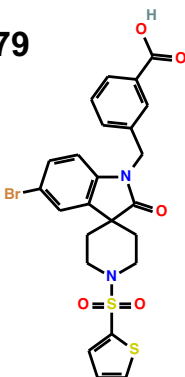
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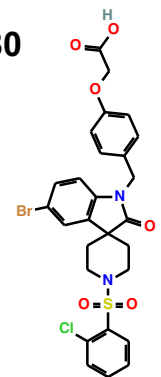
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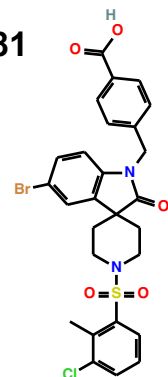
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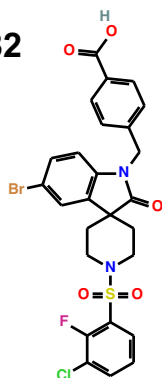
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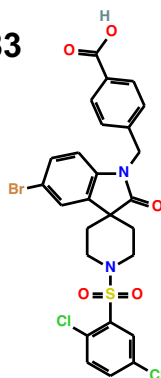
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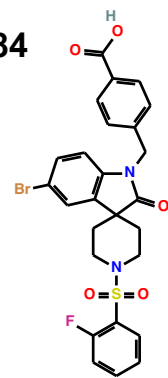
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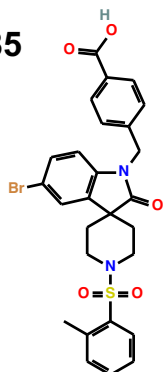
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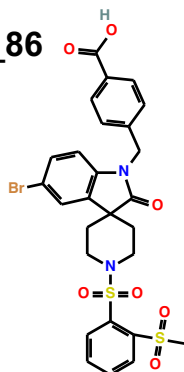
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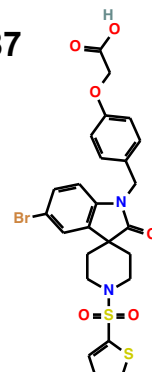
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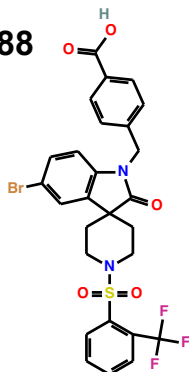
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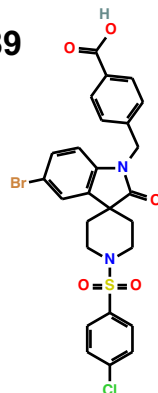
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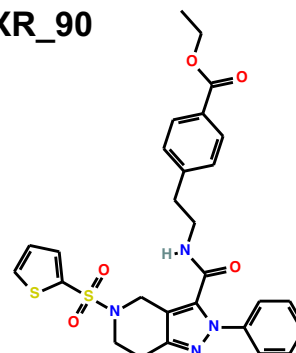
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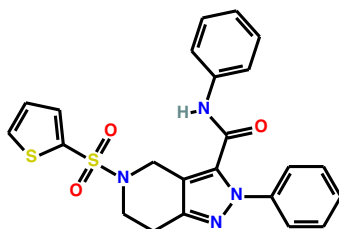
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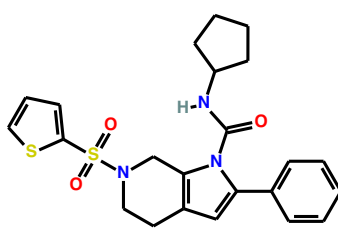
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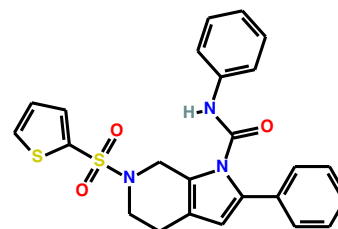
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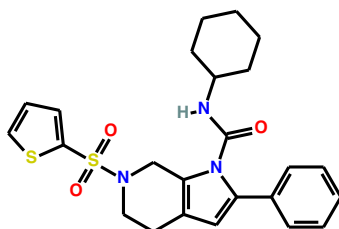
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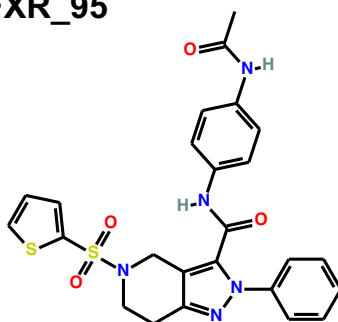
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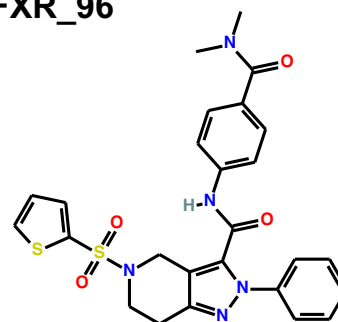
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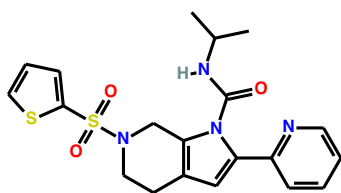
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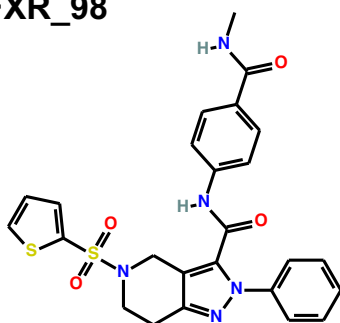
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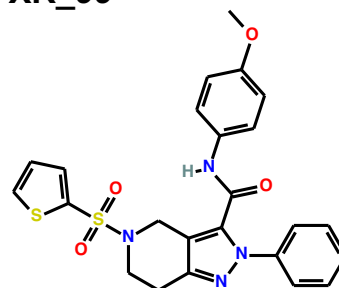
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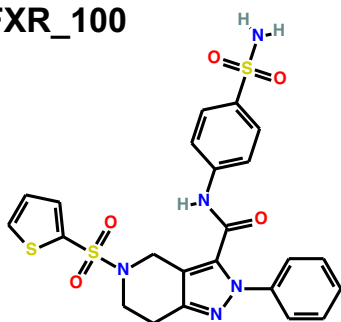
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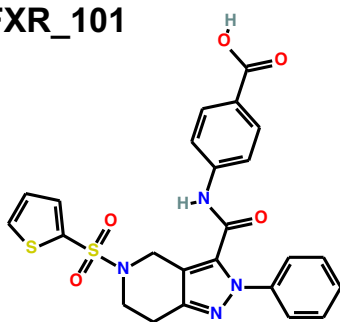
FXR_99



FXR_100



FXR_101



FXR_102

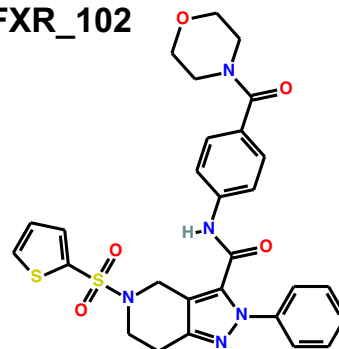


Figure S2. Thermodynamic cycle for the calculation of relative binding affinities of ligands L_1 and L_2 compared with the protein P .

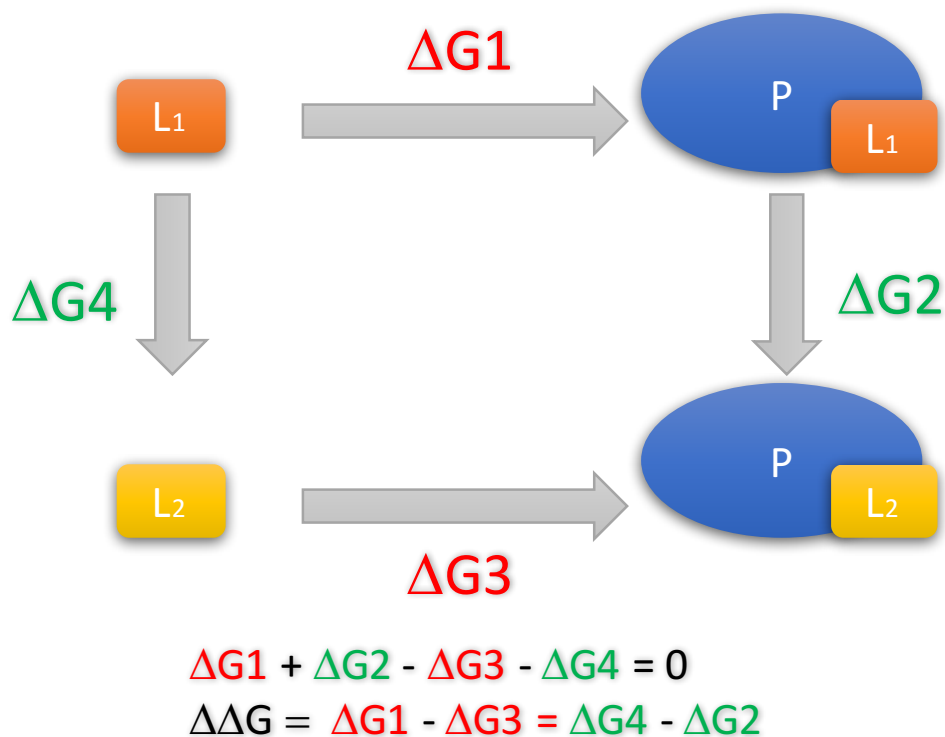


Figure S3. Schematic representation of the system used for the calculation of relative binding affinities of ligands L_1 and L_2 for the protein P , in the case of a charge conserving structural change on the ligand.

Charge conserving structural change for ligand

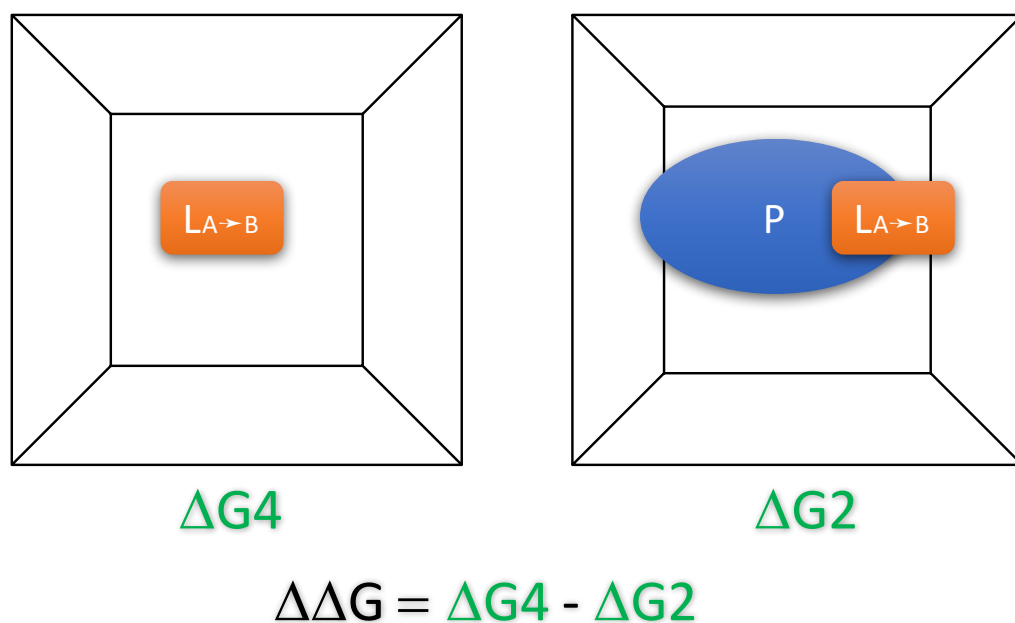


Figure S4. Schematic representation of the system used for the calculation of relative binding affinities of ligands L_1 and L_2 for the protein P , in the case of a charge modifying structural change on the ligand.

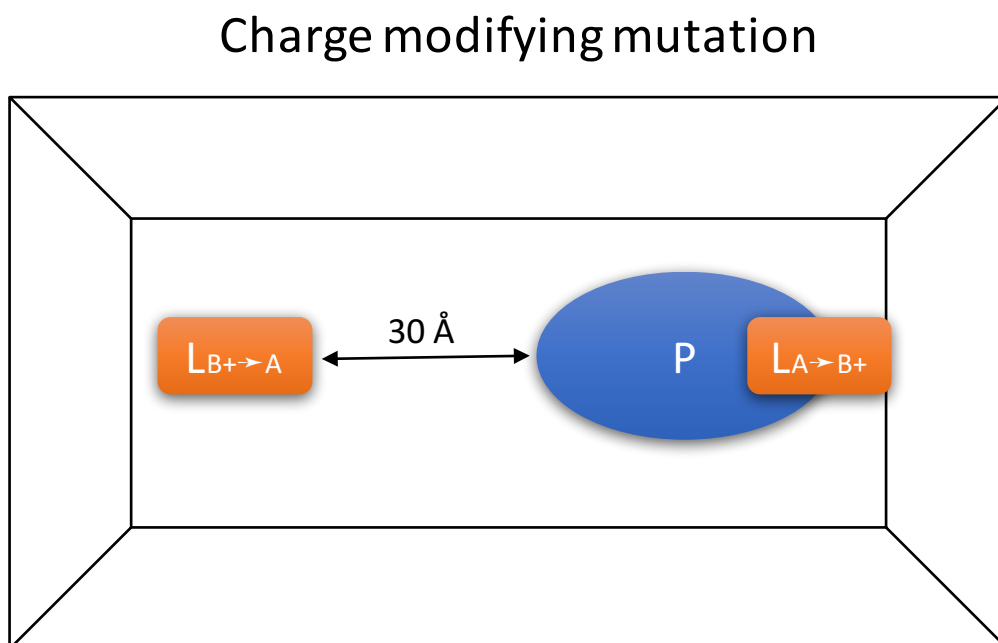
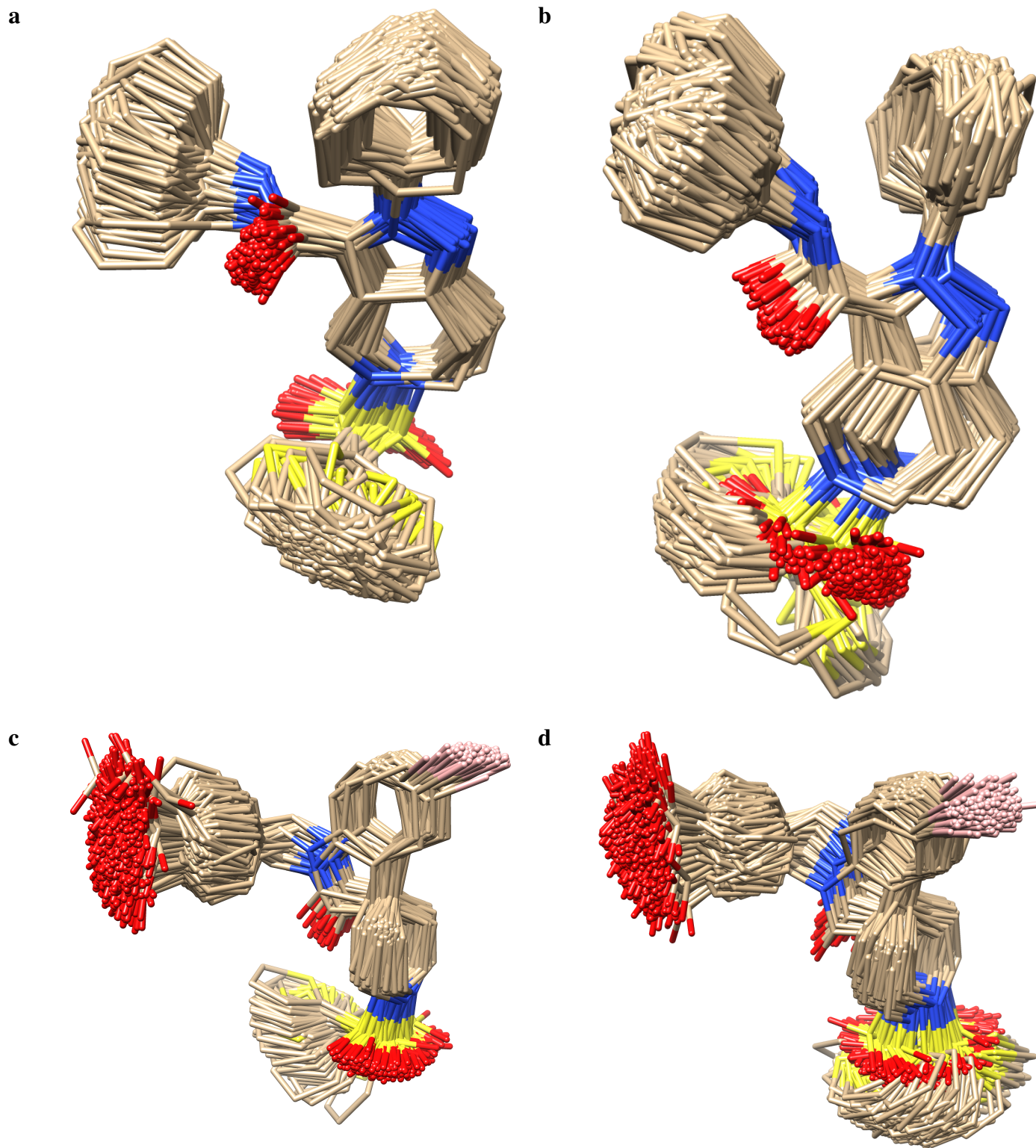
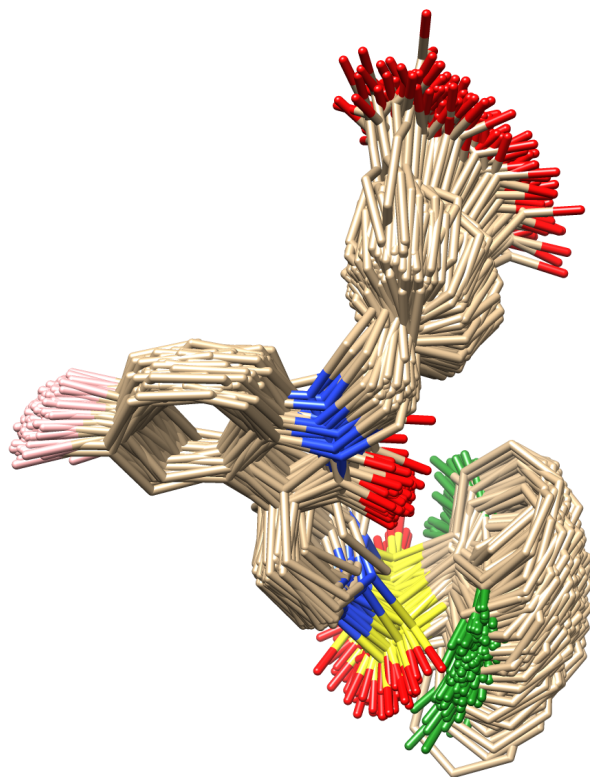


Figure S5. Conformational distribution of ligands FXR_17 (a, b), FXR_10 (c, d) and FXR_12 (e, f), as representative structures for set1 and set2. In each case, all 501 conformers extracted from the 10 ns molecular dynamics simulation of the ligand alone in water, using the OPLS-AA (a, c, e) and AMBER/GAFF (b, d, f) force fields, are represented. Hydrogen atoms are not shown for more clarity.



e



f

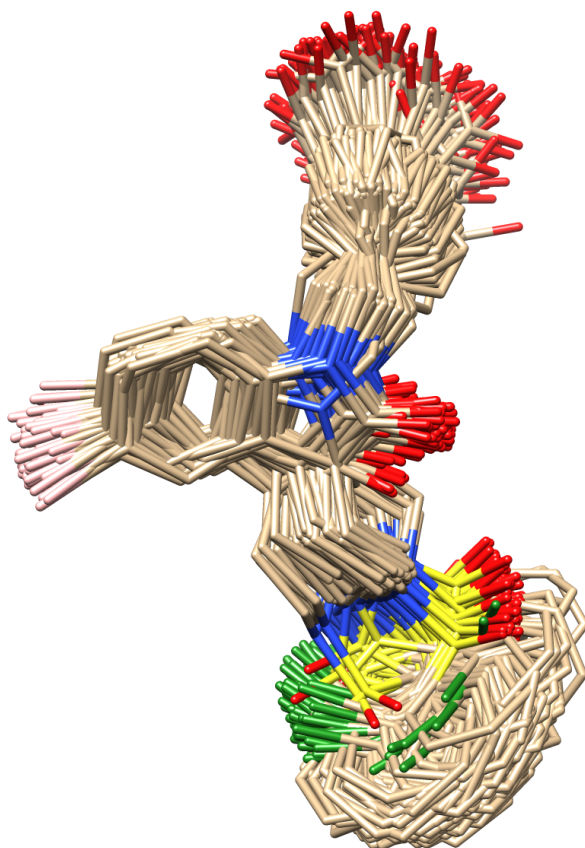


Table S1. Rank of the best RMSD poses. When no reference structure was available, the score of the first ranked pose was reported for the two submissions, therefore no rank of best RMSD pose is considered.

Ligand	Score of first ranked pose	Score of best RMSD pose	Rank of best RMSD pose (out of 10 poses)
FXR_1	57.25	57.25	—
FXR_2	57.27	57.27	—
FXR_3	59.03	59.03	—
FXR_4	63.35	64.13	6
FXR_5	58.53	62.45	7
FXR_6	49.10	52.44	4
FXR_7	48.07	49.36	4
FXR_8	53.28	54.13	3
FXR_9	43.55	43.55	1
FXR_10	51.19	51.19	—
FXR_11	44.97	44.97	—
FXR_12	47.66	47.66	—
FXR_13	41.27	41.27	1
FXR_14	45.85	46.23	2
FXR_15	49.65	49.65	—
FXR_16	56.47	56.47	—
FXR_17	42.05	42.05	—
FXR_18	54.34	54.34	—
FXR_19	49.24	52.23	9
FXR_20	48.05	49.73	2
FXR_21	46.07	46.26	2
FXR_22	51.95	51.95	1
FXR_23	53.66	57.20	10
FXR_24	44.42	45.46	7
FXR_25	46.74	47.83	7
FXR_26	39.43	39.43	1
FXR_27	39.50	41.40	5
FXR_28	40.42	42.27	7
FXR_29	41.16	43.78	6
FXR_30	41.54	44.58	7
FXR_31	44.30	45.09	4
FXR_32	52.50	53.19	2
FXR_33	40.94	42.95	5
FXR_34	52.87	59.51	10
FXR_35	40.64	40.64	1
FXR_36	32.84	35.97	7
FXR_37	55.97	58.21	10
FXR_38	50.16	50.16	—
FXR_39	51.58	51.66	4
FXR_40	48.89	51.89	5
FXR_41	45.89	45.89	—
FXR_42	45.92	50.25	7
FXR_43	46.24	46.24	—
FXR_44	48.13	48.13	—
FXR_45	39.38	39.38	—
FXR_46	42.54	42.54	—
FXR_47	51.05	51.05	—
FXR_48	43.75	43.75	—
FXR_49	43.65	43.65	—
FXR_50	52.93	54.39	6
FXR_51	48.25	48.74	2
FXR_52	45.76	54.79	3
FXR_53	39.81	41.12	7

FXR_54	46.19	48.11	4
FXR_55	45.06	45.37	4
FXR_56	43.69	43.69	1
FXR_57	47.05	48.29	8
FXR_58	38.19	38.19	1
FXR_59	39.35	41.05	4
FXR_60	41.27	43.93	2
FXR_61	40.38	43.31	5
FXR_62	38.54	38.63	7
FXR_63	40.07	40.55	9
FXR_64	39.42	41.64	4
FXR_65	38.25	40.65	7
FXR_66	41.96	43.83	5
FXR_67	36.46	37.87	2
FXR_68	41.87	44.72	3
FXR_69	33.62	33.66	2
FXR_70	39.28	45.34	6
FXR_71	36.91	38.68	7
FXR_72	38.51	38.77	2
FXR_73	49.38	49.38	–
FXR_74	48.03	48.03	–
FXR_75	50.52	50.52	–
FXR_76	48.20	48.20	–
FXR_77	44.43	44.43	–
FXR_78	46.49	46.49	–
FXR_79	48.64	48.64	–
FXR_80	37.81	37.81	–
FXR_81	44.02	44.02	–
FXR_82	45.46	45.46	–
FXR_83	45.72	45.72	–
FXR_84	47.15	47.15	–
FXR_85	46.19	46.19	–
FXR_86	44.59	44.59	–
FXR_87	41.90	41.90	–
FXR_88	41.68	41.68	–
FXR_89	47.94	47.94	–
FXR_90	41.55	41.55	–
FXR_91	48.44	48.44	–
FXR_92	55.18	55.18	–
FXR_93	49.39	49.39	–
FXR_94	53.68	53.68	–
FXR_95	41.40	41.40	–
FXR_96	42.61	42.61	–
FXR_97	55.88	55.88	–
FXR_98	42.50	42.50	–
FXR_99	45.73	45.73	–
FXR_100	40.67	40.67	–
FXR_101	45.12	45.12	–
FXR_102	40.80	40.80	–
Mean rank of best RMSD pose (from 53 values)			4.68

Table S2. Tanimoto similarity matrix for the compounds belonging to set1.

Tanimoto	FXR _17	FXR _45	FXR _46	FXR _47	FXR _48	FXR _49	FXR _91	FXR _93	FXR _95	FXR _96	FXR _98	FXR _99	FXR _100	FXR _101	FXR _102	Mean value per compound
FXR_17	1.00	0.91	0.88	0.95	0.93	0.87	0.84	0.68	0.85	0.88	0.88	0.86	0.84	0.94	0.87	0.88
FXR_45	0.91	1.00	0.81	0.88	0.85	0.81	0.78	0.63	0.78	0.81	0.81	0.91	0.77	0.86	0.80	0.83
FXR_46	0.88	0.81	1.00	0.86	0.91	0.97	0.95	0.76	0.94	0.98	0.98	0.83	0.95	0.90	0.94	0.91
FXR_47	0.95	0.88	0.86	1.00	0.93	0.86	0.86	0.69	0.86	0.86	0.86	0.88	0.86	0.90	0.85	0.87
FXR_48	0.93	0.85	0.91	0.93	1.00	0.91	0.90	0.73	0.90	0.90	0.90	0.85	0.89	0.90	0.89	0.89
FXR_49	0.87	0.81	0.97	0.86	0.91	1.00	0.93	0.76	0.93	0.97	0.97	0.83	0.93	0.88	0.92	0.90
FXR_91	0.84	0.78	0.95	0.86	0.90	0.93	1.00	0.78	0.97	0.94	0.94	0.85	0.98	0.86	0.90	0.90
FXR_93	0.68	0.63	0.76	0.69	0.73	0.76	0.78	1.00	0.77	0.76	0.75	0.68	0.77	0.69	0.73	0.75
FXR_95	0.85	0.78	0.94	0.86	0.90	0.93	0.97	0.77	1.00	0.94	0.94	0.85	0.97	0.85	0.89	0.90
FXR_96	0.88	0.81	0.98	0.86	0.90	0.97	0.94	0.76	0.94	1.00	0.98	0.82	0.93	0.89	0.94	0.91
FXR_98	0.88	0.81	0.98	0.86	0.90	0.97	0.94	0.75	0.94	0.98	1.00	0.82	0.93	0.89	0.93	0.91
FXR_99	0.86	0.91	0.83	0.88	0.85	0.83	0.85	0.68	0.85	0.82	0.82	1.00	0.85	0.83	0.80	0.84
FXR_100	0.84	0.77	0.95	0.86	0.89	0.93	0.98	0.77	0.97	0.93	0.93	0.85	1.00	0.86	0.89	0.89
FXR_101	0.94	0.86	0.90	0.90	0.90	0.88	0.86	0.69	0.85	0.89	0.89	0.83	0.86	1.00	0.85	0.87
FXR_102	0.87	0.80	0.94	0.85	0.89	0.92	0.90	0.73	0.89	0.94	0.93	0.80	0.89	0.85	1.00	0.88
Global mean value																0.88

Table S3. Tanimoto similarity matrix for the compounds belonging to set2.

Tanimoto	FXR _10	FXR _12	FXR _38	FXR _41	FXR _73	FXR _74	FXR _75	FXR _76	FXR _77	FXR _78	FXR _79	FXR _81	FXR _82	FXR _83	FXR _84	FXR _85	FXR _88	FXR _89	Mean value per compound
FXR_10	1.00	0.85	0.94	0.81	0.87	0.89	0.75	0.90	0.82	0.83	0.95	0.83	0.84	0.83	0.89	0.89	0.87	0.85	0.87
FXR_12	0.85	1.00	0.81	0.94	0.75	0.92	0.64	0.94	0.96	0.97	0.82	0.95	0.96	0.97	0.92	0.92	0.91	0.97	0.90
FXR_38	0.94	0.81	1.00	0.85	0.83	0.84	0.74	0.85	0.78	0.79	0.91	0.80	0.80	0.79	0.84	0.85	0.83	0.81	0.84
FXR_41	0.81	0.94	0.85	1.00	0.71	0.87	0.63	0.89	0.91	0.92	0.78	0.91	0.91	0.92	0.87	0.88	0.86	0.92	0.87
FXR_73	0.87	0.75	0.83	0.71	1.00	0.77	0.75	0.79	0.72	0.73	0.88	0.73	0.73	0.73	0.77	0.77	0.76	0.75	0.78
FXR_74	0.89	0.92	0.84	0.87	0.77	1.00	0.66	0.98	0.89	0.90	0.85	0.90	0.91	0.90	0.96	0.96	0.95	0.92	0.89
FXR_75	0.75	0.64	0.74	0.63	0.75	0.66	1.00	0.67	0.62	0.62	0.74	0.62	0.63	0.62	0.66	0.66	0.65	0.64	0.68
FXR_76	0.90	0.94	0.85	0.89	0.79	0.98	0.67	1.00	0.90	0.92	0.86	0.91	0.92	0.91	0.98	0.98	0.96	0.94	0.91
FXR_77	0.82	0.96	0.78	0.91	0.72	0.89	0.62	0.90	1.00	0.95	0.79	0.93	0.94	0.96	0.89	0.89	0.88	0.94	0.88
FXR_78	0.83	0.97	0.79	0.92	0.73	0.90	0.62	0.92	0.95	1.00	0.80	0.93	0.94	0.96	0.90	0.90	0.89	0.95	0.88
FXR_79	0.95	0.82	0.91	0.78	0.88	0.85	0.74	0.86	0.79	0.80	1.00	0.80	0.81	0.80	0.85	0.85	0.84	0.82	0.84
FXR_81	0.83	0.95	0.80	0.91	0.73	0.90	0.62	0.91	0.93	0.93	0.80	1.00	0.95	0.94	0.90	0.93	0.90	0.95	0.88
FXR_82	0.84	0.96	0.80	0.91	0.73	0.91	0.63	0.92	0.94	0.94	0.81	0.95	1.00	0.95	0.94	0.91	0.91	0.96	0.89
FXR_83	0.83	0.97	0.79	0.92	0.73	0.90	0.62	0.91	0.96	0.96	0.80	0.94	0.95	1.00	0.90	0.90	0.88	0.95	0.88
FXR_84	0.89	0.92	0.84	0.87	0.77	0.96	0.66	0.98	0.89	0.90	0.85	0.90	0.94	0.90	1.00	0.96	0.97	0.92	0.90
FXR_85	0.89	0.92	0.85	0.88	0.77	0.96	0.66	0.98	0.89	0.90	0.85	0.93	0.91	0.90	0.96	1.00	0.97	0.92	0.90
FXR_88	0.87	0.91	0.83	0.86	0.76	0.95	0.65	0.96	0.88	0.89	0.84	0.90	0.91	0.88	0.97	0.97	1.00	0.91	0.89
FXR_89	0.85	0.97	0.81	0.92	0.75	0.92	0.64	0.94	0.94	0.95	0.82	0.95	0.96	0.95	0.92	0.92	0.91	1.00	0.90
Global mean value																			0.86

General conclusions and **future perspectives**

General conclusions and future perspectives

Antimicrobials are a cornerstone of modern medicine. They have shaped medicine and helped save countless lives since their discovery [Aminov, 2010]. They are fundamental for treatment of infections. However, their use, misuse, and abuse have led to the selection and spread of resistance mechanisms [O'Neill, 2014], and nowadays, already, multi- and extensively-drug-resistant pathogens are threatening with returning medicine to a pre-antibiotics era [Basak et al., 2016]. The fight against antimicrobial resistance (AMR) must be tackled from different angles, and defeating it may even require a change in strategy, adopting new alternatives such as phage therapy for example [Kakasis and Panitsa, 2018]. However, their efficacy is unquestioned and therefore, attempts to diminish the spread of AMR, develop new antimicrobials against novel or already exploited targets, and develop new efficient inhibitors against current AMR mechanisms, are promising options. The efficacy and safety of β -lactams have placed them as the first antimicrobial therapy option, and therefore tackling resistance against them is an interesting subject. The development of novel antimicrobials targeting PBPs is interesting because of the clinical safety of these drugs due to the lack of similar targets in the human body. And given the main resistance mechanism is the acquisition and expression of β -lactamases, targeting these is another interesting strategy, because it would allow us to keep benefiting from the large arsenal of safe β -lactam drugs already available today.

Both these strategies are likely to encounter better results if approached with a rational strategy, and therefore the study of β -lactamases provides valuable information to gain insights into their structure-function relationship and their evolution. This may allow us to design drugs that escape the currently widespread resistance mechanisms as well as the next novel mutants that could emerge. These drugs need to inhibit a wider range of β -lactamases in order to improve therapeutic options, and design better antimicrobial-inhibitor combinations less likely to fail against novel mutations that can result in β -lactamases resistant to inhibition while still effective against antimicrobials [Shields et al., 2017]. Meanwhile, the usefulness of molecular modelling techniques to study these topics can be further evaluated and improved. Their competence to model kinetic parameters for these enzymes and substrates or inhibitors can be studied and improved, and methods can be combined to arrive faster to results and using less computational resources. Accordingly, organizing the publicly available data in a combined, ordered and efficient manner in a database allows for the productive exploitation of it in an easier and faster way, and may even reveal information not previously recognized.

The studies on the novel class enzyme C CMY-136 reveal how unusual mutations in very conserved positions can still be selected in β -lactamase, expanding the resistance profile of CMY-2, and conferring resistance to a relatively new treatment such as ceftolozane/tazobactam. Structural characterization of this enzyme suggests that the expanded spectrum of the enzyme may not be a direct consequence of an interaction between the Y221H substitution and the substrates, but of the conformational change on a

conserved structural feature of this family, the Ω loop, caused by this mutation. Mutations expanding the hydrolysis spectrum of class C enzymes by altering the characteristics of this loop had already been recognized [Crichlow et al., 1999; Kotsakis et al., 2011]. The structure of CMY-136 suggests that the folding and conserved interactions of the Ω loop are disrupted (e.g. the pi-pi stacking of residues Y199, W201, H210 or the hydrogen bonds between the Ω loop and Y199 or E61). The dynamics of these changes can also be observed using molecular dynamics (MD) simulations. Thus, recognition of such features during modelling of a novel structure might be taken as hints of an expanded-spectrum. Moreover, given that this feature is conserved in all class C enzymes (presenting either a pi-pi stacking of H210 between Y199 and W201 or a cation-pi interaction of R210 between Y199 and W201), the possibility of developing molecules that target these interactions in a region not comprising the active site cavity to alter the enzyme function may be evaluated, as it has been reported for TEM-1 [Horn and Shoichet, 2004].

OXA-427 seems to derive from the chromosomal class D β -lactamase AmpS from *Aeromonas media*, but OXA-427 is located in a transferable plasmid that was recovered over a 2-year period from different species in hospital isolates in Belgium [Bogaerts et al., 2017]. Although the hydrolytic efficiencies determined do not stand out as impressive, the enzyme was reported to confer resistance to penicillins, ceftazidime, cefepime, aztreonam, and ertapenem. Therefore, this is an enzyme that can be transferred to different Enterobacteriaceae species and can already confer resistance to at least one member of each β -lactam family. Given the plasticity of β -lactamases to mutate and expand their hydrolytic profile, this novel class D β -lactamase might be considered a potentially serious threat. The structural characterization of OXA-427 reveals a class D β -lactamase with curious features, such as the hydrophobic bridge between loops α 3- α 4 and β 7- α 10, the more extended and flexible β 4- β 5 loop, or the partially carbamoylated active site lysine. These features may already be curious for being unusual, but they are also interesting because of the effect they may have on the hydrolytic profile. This might highlight, together with other examples, what to pay attention to when trying to predict if a certain structure may be able to hydrolyse a certain β -lactam family or not. Just as with OXA-48loop18 (see below), our results suggest that the flexibility and conformation of the β 4- β 5 loop may be important for class D enzymes to hydrolyse extended spectrum cephalosporins. Taking into account the fact that OXA-427 seems to bind most substrates with fairly good affinity, the hydrophobic bridge may be important in improving binding affinity for certain substrates, a role that had already been proposed in the case of OXA-24/40 [Kaitany et al., 2013]. Lastly, the partially carbamoylated lysine 54 (analogous to lysine 73 in OXA-48) in the crystal structure, in the absence of acidic conditions for crystallization or of ligands that may induce decarbamoylation, suggests that the stability of KCX may be lower for this enzyme compared to other class D β -lactamases. Studying the influence of surrounding residues on the stability of this crucial residue might point to novel interactions that might be important for the development of inhibitors for class D enzymes.

The study of the β 5- β 6 loop in OXA-48 highlights the relevance of this loop in determining the hydrolysis profile of OXA-48-like β -lactamases, and likely of many, if not all, class D enzymes as well. The wide dissemination of OXA-48 [Albiger et al., 2015; Dortet et al.,

2017], the ongoing selection of natural variants (such as OXA-517 [Dabos et al., 2018a] or OXA-163 [Poirel et al., 2011]) and the extent to which a single mutation may influence the spectrum of an enzyme (e.g. OXA-48 217Pdel mutant [Zavala et al., 2018]), illustrate the importance of evaluating the propensity of these enzymes to evolve and overcome current therapeutic options by means of biochemical and structural studies such as those performed in our group (e.g. the β 5- β 6 loop replacement [Dabos et al., 2018b] and directed mutagenesis [Dabos et al., 2018c]). Furthermore, the crystallization of the OXA-48 217Pdel mutant demonstrates how research aimed at tackling certain questions might fortuitously lead in new findings (e.g. that nitrate might act as an inhibitor of OXA-48 just as well as halogens).

Lastly, having recognized the importance of better understanding of the structure-function relationship of β -lactamases in the rational development of β -lactam drugs and β -lactamase inhibitors, it becomes clear how the phenotypical, biochemical and structural characterization of β -lactamases produces valuable data that may be exploited. However, as the amount of research done and results obtained increases, it becomes more and more difficult to learn what has already been published while at the same staying up to date with new developments, let alone compile this data into useful information to analyse, and finally derive knowledge out of it. Therefore, all the studies carried out here have benefitted greatly from the information that could be quickly retrieved from the Beta-Lactamase DataBase (BLDB) developed in our laboratory [Naas et al., 2017]. The up-to-date biochemical, functional and structural data compiled within the database and conveniently ordered has readily provided lots of information to guide and test hypotheses, as well as easy access to published results. This has been a straightforward and simple way to use the BLDB, and developing automated methods for a more comprehensive analysis of the database may lead to gaining deeper knowledge in this field. Thus, the BLDB may prove to be an invaluable resource in future studies.

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Keywords: β -lactamase, X-ray structure, Molecular Dynamics.

Antimicrobial resistance (AMR) has become a major threat to public health nowadays. The use and abuse of antibiotics is increasingly leading to selection and spread of resistance mechanisms worldwide, greatly compromising our capacity to treat infectious diseases. AMR might ultimately result in a future without effective antimicrobial therapy. Due to their safety and clinical efficacy, β -lactams are the most utilized antimicrobial therapy, and the most common resistance mechanism is the expression of β -lactamases. Therefore, the development of new antimicrobial drugs, for novel or already known targets, is of utmost importance. In particular, the development of novel inhibitors towards β -lactamases is also quite promising, as it would allow us to continue using the effective and safe antimicrobial drugs already available today. The biochemical and structural study of novel β -lactamases or synthetic mutants, through X-ray crystallography and various molecular modelling techniques (homology modelling, docking, molecular dynamics, water network analysis), can provide valuable information. In this context, we have characterized phenotypically, biochemically and structurally several β -lactamases.

The CMY-136 β -lactamase possesses an unusual mutation, Y221H, as compared to CMY-2, in a position highly conserved among class C β -lactamases. Crystallographic and molecular modelling experiments reveal a steric impediment around the mutated position 221 that may affect the conformation and dynamics of the Ω -loop, and therefore account for an increased turnover rate for bulky substrates and a decreased affinity for most substrates as compared to CMY-2.

The crystal structure of the OXA-427, a novel class D carbapenemase, shows the Lys 73 only partially carbamoylated, a very unusual characteristic for this class of β -lactamases, and an unexpected hydrophobic bridge in the vicinity of the active site. Moreover, molecular dynamics simulations

revealed an extended and highly flexible β 5- β 6 loop. Altogether, these features may explain the unique hydrolytic profile determined experimentally for this enzyme.

Modifications in the β 5- β 6 loop of the OXA-48 β -lactamase (alanine scanning, systematic deletions, replacement with the β 5- β 6 loop from OXA-18) result in profound changes in the hydrolytic profile, with gradual acquisition of cephalosporinase activity and decrease of carbapenemase activity in some cases. X-ray crystallography and molecular modelling studies suggest that the altered conformation and flexibility of this loop and of adjacent regions in these mutants may allow for the better accommodation of the bulkier cephalosporins, compared to OXA-48. Additionally, water dynamics analysis highlighted changes in the water network around and inside the active site cavity that may be responsible for the lower activity towards carbapenems. Together with studies on other naturally occurring mutants, results corroborate the relevance of the β 5- β 6 loop on the substrate profile of OXA-type enzymes. Crystal structure of the OXA-48 217 Δ P mutant reveals an unexpected self-inhibited conformation induced by the presence of a nitrate ion, a previously unknown inhibitor of class D β -lactamases.

Finally, the Beta-Lactamase DataBase (BLDB, <http://bldb.eu>) developed in our laboratory is a comprehensive, manually curated public resource providing up-to-date structural and functional information on β -lactamases. It contains all reported naturally-occurring β -lactamases and synthetic mutants, together with all available 3D structures from the PDB and the phenotypical characterization. Overall, these results constitute an essential foundation for a better understanding of the structure-function relationship of β -lactamases, which may prove crucial for the future rational development of β -lactamase inhibitors.

Titre: Etudes structure-fonction des β -lactamases.

Mots clés: β -lactamase, structures aux rayons X, dynamique moléculaire.

La résistance antimicrobienne est devenue un problème majeur de santé publique. L'usage parfois abusif d'antibiotiques conduit à la sélection et à la propagation mondiale de mécanismes de résistance. Grâce à leur efficacité clinique et faible toxicité, les β -lactamines sont les antibiotiques les plus prescrits actuellement, et le mécanisme de résistance le plus répandu est l'expression de β -lactamases. Dans ces conditions, le développement de nouveaux traitements antimicrobiens, pour des cibles connues ou nouvelles, est essentiel. Plus particulièrement, le développement de nouveaux inhibiteurs des β -lactamases est très prometteur, permettant de continuer l'utilisation des antibiotiques existants. Les études biochimiques et structurales des nouvelles β -lactamases et leurs mutants synthétiques, par cristallographie aux rayons X ou par différentes techniques de modélisation moléculaire (modélisation par homologie, « docking », dynamique moléculaire, analyse du réseau des molécules d'eau) permettent une meilleure compréhension de ces enzymes. Dans ce contexte, nous avons caractérisé plusieurs β -lactamases du point de vue phénotypique, biochimique et structural.

La β -lactamase CMY-136 contient une mutation inhabituelle, Y221H, par rapport à CMY-2, dans une position qui est très conservée parmi les enzymes de classe C. Les études cristallographiques et de modélisation moléculaire ont révélé des interactions stériques défavorables autour de la position mutée 221 qui peuvent affecter la conformation et la dynamique de la boucle Ω , et qui pourraient expliquer l'hydrolyse plus efficace des substrats volumineux et l'affinité plus faible de la plupart des substrats par rapport à la CMY-2.

La structure cristallographique de la β -lactamase OXA-427, une nouvelle carbapénémase de classe D, montre une Lys73 très peu carbamoylée, ce qui est très inhabituel pour cette classe d'enzyme, ainsi qu'un pont hydrophobe à proximité du site actif. Les simulations de dynamique moléculaire ont montré que la boucle β 5- β 6 est plus flexible et dans

une conformation étendue. Ces résultats peuvent expliquer le profil d'hydrolyse unique observé expérimentalement pour cette enzyme.

Les modifications dans la boucle β 5- β 6 de la β -lactamase OXA-48 (mutations d'alanines, délétions systématiques, remplacement par la boucle β 5- β 6 de la β -lactamase OXA-18) provoquent des modifications importantes dans le profil d'hydrolyse, avec une acquisition graduelle d'une activité cephalosporinase et une diminution de l'activité carbapénémase dans certains cas. Des études de cristallographie aux rayons X et modélisation moléculaire suggèrent que les différences de conformation et de flexibilité dans cette boucle et les régions adjacentes permettent une meilleure fixation des céphalosporines plus volumineuses, par rapport à l'OXA-48. L'analyse de la dynamique des molécules d'eau dans le site actif montre des changements qui sont potentiellement responsables de la diminution de l'activité par rapport aux carbapénèmes. En complément des études sur les mutants naturels, ces résultats confirment l'importance de la boucle β 5- β 6 pour la spécificité de substrat des enzymes de type OXA-48. La structure cristallographique du mutant 217 Δ P de l'OXA-48 présente une conformation auto-inhibée inattendue, induite par la présence d'un ion nitrate, un inhibiteur auparavant inconnu des β -lactamases de classe D.

La « Beta-Lactamase DataBase » (BLDB, <http://bldb.eu>) développée dans notre équipe est une ressource publique exhaustive contenant des données relatives aux β -lactamases, vérifiées et mises à jour régulièrement. Cette base de données contient tous les mutants naturels et synthétiques de β -lactamases, ainsi que toutes les structures 3D disponibles dans la PDB et la caractérisation phénotypique.

Globalement, ces résultats représentent le prérequis pour une meilleure compréhension des relations structure-fonction des β -lactamases et pour le futur développement rationnel d'inhibiteurs pour ces enzymes.